

STANDARDIZATION OF THE HUMAN PERIPHERAL LYMPHOCYTE  
MICRONUCLEUS TEST FOR THE QUANTIFICATION  
OF GENOTOXIC EXPOSURE

by

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in

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## ABSTRACT

There is a paucity of in vitro tests to determine potential genotoxicity in human beings. In response to this need, a modified microculture system was developed in order to study micronuclei production in human peripheral blood lymphocytes. Phytohemagglutinin (PHA) was added to peripheral blood lymphocytes exposed to increasing doses of gamma radiation. Results show that neither PHA or radiation alone induce micronuclei production above background levels. However, PBML exposed to gamma radiation followed by incubation with PHA do produce micronuclei. A standardized assay measuring micronuclei, gamma interferon, and DNA production, and an interpretation of the mechanism of induction of PBML micronuclei are presented.

To the memory of my father, Saul Harold Rutstein.

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## LIST OF ABBREVIATIONS

cpm	counts per minute
FBS	Fetal Bovine Serum
<sup>3</sup> HTdr	tritiated thymidine
ug/ml	micrograms per milliliter
mn	micronuclei
PBML	Peripheral Blood Mononuclear Leukocyte
PHA	phytohemagglutinin
R	rads
SCE	Sister-Chromatid Exchange

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## PREFACE

The original proposed study for this research project was to apply a human lymphocyte micronucleus test to assess genotoxic changes due to exposure by dry cleaning workers to perchloroethylene, the dry cleaning agent used exclusively in the Salt Lake Valley. Perchloroethylene vapor is inhaled by workers throughout the dry cleaning process. Skin comes in contact with the chemical during the transfer of wet clothes from washers to dryers. The question that had been proposed was whether dry cleaning workers who were routinely exposed to perchloroethylene in a manner known to cause absorption of toxic chemicals (dermal and inhalation) would have an increased number of micronuclei in their peripheral lymphocytes; further, that the micronuclei could be shown to be produced in response to a specific exposure to a genotoxic agent.

The need to show such a cause-and-effect relationship was due, in part, to the rapid introduction of chemicals into our environment. Many chemicals are produced as by-products of manufacturing processes; others are additives to food or medicines. Most chemicals have been almost irreversibly incorporated into necessities in our highly industrialized society. The ability to monitor potential

deleterious effects requires sophisticated and selective methods at the basic science level.

Efforts to proceed with the study to measure the effect of perchloroethylene were curtailed when it became evident that criteria necessary to control and standardize the micronucleus test had not been developed. This lack of standardization, which appeared throughout the literature, was finally concluded to be due to a cross-over phenomenon in science, where methodology from one discipline is applied to the study of a new phenomenon without the knowledge of what are necessary components of the experiments. An example of this phenomenon as it applies to the micronucleus test is the importance of dose-response relationships in the induction of micronuclei with phytohemagglutinin and radiation. Only one concentration of phytohemagglutinin was used without consideration that increasing or decreasing its concentration would affect micronucleus production. This oversight could minimize, maximize, or invalidate data that claimed to identify a chemical as genotoxic.

The goal of the research was redirected to standardizing a test that was being widely applied in the identification of genotoxic agents. A test was needed that would gain wide acceptance so that each laboratory did not have its own micronucleus assay with results that could not be reproduced by other researchers because of

poor methodology. A test was needed that could truly measure genotoxic exposure in human beings with easy application to large numbers of people. A test was needed that was relatively simple to perform and was highly reproducible. The following research was performed with these goals in mind.



## 1. INTRODUCTION

### 1.1 Overview

The detailed observations in the 19th century by John Snow documenting the relationship between cholera outbreaks and sewer-contaminated water in London were the beginning of modern epidemiology (Snow, 1855). It is with great diligence that industrialized nations have established systems to protect people from common infectious sources of disease. Development and maintenance of clean water supplies are still the goal of developing nations, while industrialized nations face new threats to health in the form of chemical and physical environmental toxicities. No nation is immune from the dangers of environmental poisoning as exemplified by the extreme morbidity and mortality experienced by the residents of Bophal, India (Last, 1986).

There are two major populations under consideration in environmental and occupational medicine. The first is the working population that has potential direct or indirect exposure to hazardous agents. The second population is the often unsuspecting community at large. Contamination of water supplies due to a growing variety of toxic agents is an example of the ability of toxic

materials to affect large numbers of people who are not directly exposed to manufacturing or industrial processes.

There is a need for rapid, accurate tests to identify persons with exposure to genotoxic agents prior to the development of gross morbidity and mortality. Sister-chromatid exchange (SCE) has been used to identify potential "cancer-causing agents." Recent data suggest that the assay is not as specific as once thought (Livingston, 1985). SCE is a transposition at one locus between the sister chromatids of a chromosome and does not result in an alteration of the overall chromosome morphology (Latt and Schreck, 1980). The test is time-consuming, expensive to perform, and requires sophisticated technical expertise. These constraints have rendered the sister-chromatid exchange method impractical in field studies of large populations.

The Ames test is a measure of bacterial mutagenesis and is used to identify potential carcinogens. The test identifies back mutation to histidine independence of histidine mutants of Salmonella typhimurium and can be performed with other bacterial strains that are also repair-deficient, contain abnormalities in their cell walls that make them permeable to carcinogens, and carry an R factor, which enhances mutagenesis. The Ames test detects carcinogens by preincubating the test substance and the activation system with the test organism (Ames, et

al., 1973). This test is currently the most widely used as a rapid method to detect potential carcinogens but suffers from the limitation that it does not measure the carcinogenic potential of a substance on the human host under usual conditions of exposure. Thus, the Ames test may overestimate carcinogenic potential by identifying large numbers of substances that have little predicted effect. Generation of large lists of "potential" carcinogens is self-defeating, because large numbers cannot be adequately assessed.

Animal models are used to measure toxicity and carcinogenicity. Exceptional methods expose animals to test agents orally, topically, or parenterally (Timbrell, 1982). Treated animals are monitored for the development of illness, disease, birth defects, or other indicators of a pathological process. Evidence of adverse effects often are apparent only in animals that are treated with unrealistically high concentrations of test substances. Assays that purport to test the genotoxic potential of a substance are similar to the Ames test in that they do not readily predict the biological effects of suspected carcinogens on humans under realistic conditions of environmental exposure. Additionally, animal model systems may not accurately predict effects in humans.

Recognized inadequacies of existing genotoxic test methods led Schmid to develop an assay that utilized the

development of intracytoplasmic micronuclei in bone marrow cells of mice inoculated with test substances (Boller and Schmid, 1970; Schmid, 1975). The Micronucleus Test has gained rapid acceptance because of its simplicity, the relatively short time required to obtain results, and the practical importance of a rapid and accurate assay that is a marker of environmentally induced chromosomal damage. It was apparent from the beginning that the mouse Micronucleus Test could be a useful direct measure of human genotoxic changes if an acceptable alternative to bone marrow cells could be found (Matter and Schmid, 1971). Circulating peripheral blood lymphocytes obtained by routine venipuncture were substituted for bone marrow cells and utilized in a variety of circumstances. Unfortunately, enthusiasm for the concept fostered premature application of an incompletely characterized method. The requisite standardization of this analytical procedure to compare data between laboratories has not been forthcoming. Standardization is critically important at this time in light of the many variations in methods and results that exist between laboratories and the premature adoption of micronucleus formation by governmental regulatory agencies and independent investigators.

The human peripheral lymphocyte Micronucleus Test has been used in many forms. Investigators have continuously

modified both the method and data presentation. Currently, none of these methods has addressed centrally important issues of standardization and reproducibility. Nevertheless, the need for an in vitro measure of biological response to a genotoxic agent has stimulated continued efforts to modify and improve unstandardized assays. The following studies were undertaken in an effort to standardize the human peripheral blood lymphocyte Micronucleus Test with the intent to determine its informative value as an accurate indicator of genotoxic exposure.

## 1.2 The History of Micronuclei

Reticulocytes containing nuclear fragments that stained dense blue in Wright's stain preparations were the first identified micronuclei. These fragments were called Howell-Jolly bodies, after identification by Howell in 1891 (Howell, 1891) and Jolly in 1905 (Jolly, 1905). Howell-Jolly bodies represent chromosomal material that has been separated from the spindle during an abnormal mitosis. They are typically seen in splenectomized persons, as well as in those with hemolytic anemia, hyposplenism, and megaloblastic anemia (Cecil, 1985).

The prevalent hypothesis, based primarily on indirect evidence, is that micronuclei are formed from acentric chromosomal fragments that occur as a consequence of chromosomal breaks or spindle dysfunction (Countryman and

Heddle, 1976). During mitosis chromosomes proceed to spindle formation, where nuclear material lags behind. Chromatin-containing material condenses into small micronuclear bodies following separation into two daughter cells. Micronuclei can be induced with a variety of physical and chemical agents whose mode of action appear to support this mechanism of formation (Heddle, et al., 1978). Several investigators have suggested that the human mitogen-induced PBML micronucleus is formed following the first division. Thereafter, the cell dies, since the loss of nuclear material through the formation of a micronucleus is incompatible with survival (Heddle and Carrano, 1977).

The presence of micronuclei as an expression of chromosomal breakage induced by toxic agents was studied by Schmid (1975) and others (Matter and Schmid, 1971; Countryman and Heddle, 1976; Heddle, et al., 1978; Maier and Schmid, 1976) in the 1970s. The early studies were performed with intraperitoneal or subcutaneous injections of test substances into mice, followed by examination of the bone marrow extracted from the femur. Micronuclei were observed in polychromatic erythroblasts on freshly prepared slides. Schmid was encouraged by the simplicity of the assay, although he warned about technical problems, including variation in yield of bone marrow cells and subjectivity in quantitating micronuclei due to staining

artifacts, and lack of reliability in the quality of the preparations.

The mouse became the animal of choice, because of the ease of handling, abundance of animals, and low cost. Heddle reports that the spontaneous frequency of micronuclei formation varies with species. Micronucleated polychromatic erythroblasts in normal mice range between 1% and 3%, while frequency in rats ranges between 2% and 6% (Heddle, et al., 1983). Spontaneous frequencies in the murine system above 3% are suspected to be the result of technical errors within the laboratory.

Mice have been used to evaluate the ability of numerous chemicals to induce micronuclei. Schlegel and MacGregor (Schlegel and MacGregor, 1982) injected triethylene-malamine intraperitoneally into mice and demonstrated that the micronuclei that were induced remained in the circulating blood for an average of 30 days, and that there appeared to be no effective mechanism within the mouse for the removal of micronucleated erythrocytes. King and Wild (1984) evaluated transplacental mutagens for their ability to produce micronuclei in the fetus. Cyclophosphamide, procarbazine, trenimon, and mitomycin-C were separately injected into pregnant mice. Following a 26-hour incubation, the mice were sacrificed, fetal blood cells were pooled, and

polychromatic erythrocytes were examined for the presence of micronuclei. Dose-dependent increases in micronucleated erythrocytes were observed with all four agents, with up to 30% to 40% micronucleated cells.

Application of the micronucleus assay to a human system expanded its versatility by allowing the study of peripheral blood mononuclear leukocytes from persons with genetic diseases and environmental exposures to known and suspected carcinogens and genotoxins. The technique additionally permitted the study of in vitro exposures for toxic agents.

Normally, PBML are nondividing and micronuclei cannot be found in nonproliferating cells. This obstacle can be overcome, since PBML can be induced to blast transform and divide when they are exposed to the addition of mitogens such as phytohemagglutinin (PHA) in vitro. Unlike the murine system, the use of human PBML system requires much greater standardization, because of the need to culture the cells for up to 96 hours in a sterile, physiologic environment. The murine system examined cells immediately following removal from the mouse and did not use mitogens to induce proliferation. Researchers using the human mitogen-induced PBML system independently modified their methods. Such modifications often emphasized ease of performance but ignored issues of reproducibility. A brief recounting of the extremes of some of these



methodologic changes illustrates their confounding nature on reproducibility and, ultimately, on the interpretability of results.

In some cases PHA was added to unfractionated anticoagulated blood. Cells were not counted and the amount of PHA varied (Pincu, et al., 1984; Fenech and Morley, 1985a; Hogstedt, 1984; Fenech and Morley, 1985b). Other investigators separated whole blood into erythrocyte and PBML fractions on a Ficoll-Hypaque gradient. Isolated PBML were divided into two aliquots and placed in culture in volumes ranging from 1 ml to 10 ml. The actual number of PBML was unknown, since cells were not counted prior to being placed in culture. A variety of staining techniques were used (Pincu, et al., 1984; Hogstedt, 1984). Heddle began using a hypotonic saline solution that not only made micronucleated cells more visible but, also, increased the amount of cell debris and artifacts in the culture (Heddle, et al., 1983). Countryman and Heddle treated cultured PBML with radiation and mitomycin-C (1976). Increased numbers of micronuclei were reported when PBML were obtained from patients following diagnostic x-ray, and another study by Stich demonstrated increased frequency of micronuclei in the buccal mucosa of smokers (Stich and Rosin, 1983).

Phytohemagglutinin, which is a mitogen and by definition causes lymphocytes to undergo blast

transformation and divide, was added in arbitrary concentrations. The belief seemed to be that any concentration that induced blast transformation and subsequent cell division would be adequate. Implied in the rationale of inducing cells to divide is that there is a measurable increase in DNA synthesis in cells incubated with PHA. Use of arbitrary PHA concentrations can result in misrepresenting the potency of a genotoxin. Both inadequate and excessive PHA can underestimate genotoxic potential. Therefore, it is necessary to standardize the use and applications of this substance.

### 1.3 Phytohemagglutinin as a Mitogen

Phytohemagglutinin (PHA) is a mitogen extracted from the seeds of Phaseolus vulgaris, the red kidney bean. PHA was employed in early lymphocyte research to separate white cells from whole blood by its powerful and almost irreversible agglutinating properties. Nowell later discovered that PHA induced blast transformation of peripheral blood lymphocytes, many of which proceeded on to mitosis and complete cell division (Nowell, 1960). PHA is currently available in a range of purities. The most widely used is the reagent-grade lyophilized preparation, which is relatively inexpensive. PHA is also available in a highly purified form, which is more expensive than the reagent-grade material. The recommended concentration of the lyophilized preparation, 900 ug/ml, is for chromosome

analysis (Li and Osgood, 1949) and is excessive for the induction of micronuclei. Concentrations of PHA used in the many reported micronucleus assays range from a 1% solution to 5 ug/ml, and a few papers do not identify the concentration of PHA used (King and Wild, 1984; Stich and Rosin, 1983). It has been reported that micronuclei can be induced with a wide range of concentrations of PHA, and micronuclei can be induced in cultures of whole blood as well as gradient-separated PBML. Peak micronucleus production is reached between 72 and 96 hours (Fenech and Morley, 1985a).

To confirm that the concentration of PHA contributes to the induction of micronuclei and that technical variations in methodology may misrepresent the true response requires systematic testing. An understanding of the classic dose-response relationship is important in structuring experiments that will compare responses to treatments and exposures. Application of the human mitogen-induced PBML micronucleus assay without a standardized and reproducible method is premature and misleading.

Current methods differ significantly in important fundamental processes that are assumed to have been standardized prior to common use of any procedure. Major differences exist in culture conditions, volumes, numbers

of cells, concentration, and purity of the critical stimulant. Curiously, despite major procedural differences, cell assays result in the production of the same numbers of micronuclei.

#### 1.4 Culture Conditions

The criteria necessary for an assay to be reproducible are the following: First, a standard concentration of cells must be cultured in a standard volume. Second, all reagents must be of standard purity. Third, a number of concentrations have to be examined in order to identify a dose-response range and maximal stimulatory conditions for each experiment. Fourth, each variable must have a control. Fifth, comparable methods of measuring outcome must be clearly established, which include culture times, culture conditions, and concentrations of reagents.

No reports were found in the literature that adequately document the influence of the concentration of PHA on the production of micronuclei. Obviously, the concentration of PHA that is chosen must represent one that produces maximum micronuclei formation but does not produce toxicity or cell death. The dose, derived through experimentation, can be used with confidence in subsequent tests. The induction of micronuclei in human peripheral blood mononuclear leukocytes (PBML) is possible under a variety of culture conditions. The task is to standardize

the conditions under which the maximum numbers of micronuclei can be produced in order to reliably apply the test. Almost without exception, each new investigator has found reason to vary the test conditions. Modifications have rarely, if ever, been tested for reproducibility, comparability, or their relationship to previous methods or published data.

#### 1.5 Other Relevant Measures of Peripheral Blood Mononuclear Leukocyte (PBML) Function

A number of tests have been used routinely to measure PBML functions. Historically, measurements of the incorporation of tritiated thymidine into newly synthesized DNA has been used most frequently to measure lymphocyte function. Lymphokine production, such as the synthesis of gamma interferon, also is used frequently to assess PBML reactivity. In general, most PBML macro-culture systems have been replaced by microculture techniques, which accommodate greater numbers of samples. Microcultures also reduce the numbers of cells and volume of reagents used in experiments. The utilization of Ficoll-Hypaque to achieve gradient separation of PBML has yielded highly purified preparations of cells for culture.

At the time these studies were undertaken, micronucleus production had the stated potential of being widely applied to the study of human genotoxic exposure but was limited by cumbersome methodologies, an absence of

standardization, and low numbers of micronuclei that could be demonstrated. Based on its appeal as a potential successor of the more technically difficult SCE assay, the Micronucleus Test was already in the process of being widely applied to the study of a number of important problems and processes. Preventive application of multiple potentially noncomparable assays produced data that were not comparative. The potential existed for the development of a body of knowledge comprised of the findings of individual experiments whose results could not be compared or verified. Standardization, although an amazingly simple concept, had not been developed and threatened the progress and credibility of this important area of research.

## 2. MATERIALS AND METHODS

### 2.1 Isolation of Peripheral Blood Mononuclear Leukocytes (Boyum, 1968)

#### Materials:

Pan-heparin, preservative free, 10,000 units/ml,  
Abbott Laboratories, Chicago, Illinois  
30 milliliter plastic syringe with 20-gauge needle  
RPMI-1640 medium, Gibco Laboratories, Grand Island,  
New York  
Fetal Bovine Serum (FBS), Hyclone, Logan, Utah  
Ficoll-Hypaque solution, Sigma Pharmaceuticals

#### Method:

Venipuncture is used to obtain 30 ml of heparinized blood. The blood-filled syringe is gently rotated to prevent clotting, the needle is removed, and the blood gently expressed into a sterile 100 ml glass bottle. Whole blood is diluted with an equal volume of RPMI-1640 and swirled. The diluted blood is carefully layered on top of the Ficoll-Hypaque in a ratio of 3:1 blood to Ficoll-Hypaque, in 15 or 50 ml sterile centrifuge tubes. The tubes are placed in a Beckman, model TJ-6 table-top centrifuge at 1500 rpm for 30 minutes. Centrifugation produces a gradient of blood products with the mononuclear leukocytes (lymphocytes and monocytes) concentrated in a

band at the interface above the red blood cells (Boyum, 1968). These leukocytes are removed with a 5 ml pipet and placed in a 50 ml centrifuge tube. The mononuclear leukocytes are diluted and washed twice with RPMI-1640 containing 17% fetal bovine serum. Following the second wash, the PBML are resuspended in 10 ml of complete medium (RPMI-1640 with 17% FBS) and counted with a hemocytometer. PBML are diluted to a concentration of  $2 \times 10^6/\text{ml}$  in complete medium.

## 2.2 Peripheral Blood Mononuclear Leukocyte Isolation and Micronucleus Assay (Livingston, 1985)

This method was in general use in the laboratory at the time the current project was initiated.

### Materials:

Phytohemagglutinin-PHA-M, Burroughs-Wellcome

RPMI-1640, Gibco Laboratory, Grand Island, New York

Fetal Bovine Serum, Gibco Laboratory, Grand Island, New York

### Method:

Whole blood is obtained by venipuncture with a green-topped vacutainer tube. The blood is rotated to prevent clotting. The cap is removed and the blood is diluted with an equal volume of RPMI-1640. The diluted blood is pipetted into 15 ml centrifuge tubes in equal volumes. Ficoll-Hypaque is gently introduced below the blood through a spinal needle attached to a 20 ml syringe.



The tubes are centrifuged at 1800 rpm for 30 minutes. The concentrated PBML band at the interface above the red blood cells is removed with a 5 ml pipet and placed in a new centrifuge tube. These PBML are then washed twice with RPMI-1640 containing 17% FBS. Following the second wash, the cell pellet is suspended in 10 ml complete medium containing PHA. The resuspended cells are not counted but are divided into two equal aliquots in 15 ml centrifuge tubes to which another 5 ml of complete medium containing PHA is added to each tube. The tubes are capped tightly and placed at an angle in a 37°C incubator.

Following an 88-hour incubation, the cells are concentrated in a pellet in their tubes by centrifugation at 1000 rpm for 15 minutes. The supernatant medium is aspirated, leaving just enough medium to resuspend the pellet. Cytocentrifuge slides are prepared by combining 2 to 5 drops of cells from a Pasteur pipet, 1 drop of glycerol, and 2 drops of phosphate-buffered saline (PBS, pH 7.45). The slides are spun in a table-top cytocentrifuge at 6500 rpm for 5 minutes.

The slide preparations are dehydrated by being immersed in methanol for 3 minutes, followed by immersion in Wrights-Giemsa stain for 5 minutes and, then, buffer-stain solution for 12 minutes. The slides are gently washed under tap water, air-dried for 24 hours, and mounted with Permunt and coverslips.

### 2.3 Quantifying Micronuclei (Livingston, 1985)

#### Method:

Stained slides are microscopically examined under an oil immersion objective at 1200X. The entire slide is scanned, but only fields containing a micronucleated cell are counted. This procedure is repeated until 1000 total cells are counted. Duplicate slides are prepared from each culture and the results are expressed as the mean of the duplicates/1000 cells.

### 2.4 Microculture of Peripheral Blood Mononuclear Leukocytes--Modified

#### Materials:

96-well microtiter plates, Falcon, Microtest II,  
Fisher Scientific  
Phytohemagglutinin, HA-15, Burroughs-Wellcome  
Beckenham, England  
Gammeter, Isomedix, Inc., Parsippany, New York

#### Method:

PBML are isolated according to the method of Boyum in Section 2.1. The cells are then divided into 4 aliquots for gamma radiation exposure. A cesium 451 source is utilized to irradiate the cells. The source is calibrated to deliver 480 rads/min. PBML at their final concentration of  $2 \times 10^6$ /ml in 4 ml aliquots are dispensed into sterile glass vials and irradiated as follows:

- a. 200 rads = 0.416 minutes
- b. 100 rads = 0.208 minutes
- c. 50 rads = 0.104 minutes
- d. 0 rads = control

Using a 1 ml pipet, 0.1 ml volumes containing  $2 \times 10^6$ /ml PBML are added to each well of a Falcon microtiter plate. PHA, supplied as a 5 mg bottle lyophilized product, is reconstituted with RPMI-1640 without serum. This stock solution is then used to make all subsequent dilutions. Aliquots of the PHA solution are stored at  $-70^\circ\text{C}$ . Each 1 ml aliquot is thawed no more than twice in order to diminish potential loss of activity of the PHA. Final concentrations of PHA utilized in the experiments range from 3-400 ug/ml. The volume of PHA added to each well is 0.1 ml, regardless of the concentration. Control cultures receive 0.1 ml of complete medium, RPMI-1640 containing 17% FBS, without PHA. Figure 1 describes the procedural steps described in this section.

The microculture plates have 8 horizontal rows of 12 wells each, as diagrammed in Figure 2. Thus, each row contains an entire PHA dose curve, plus a cell control. Two microculture plates are required for these multidose experiments. Continuing in this fashion, the first 3 horizontal rows are established with cells that are nonirradiated controls; the next 3 rows are cells that

## LYMPHOCYTE ISOLATION

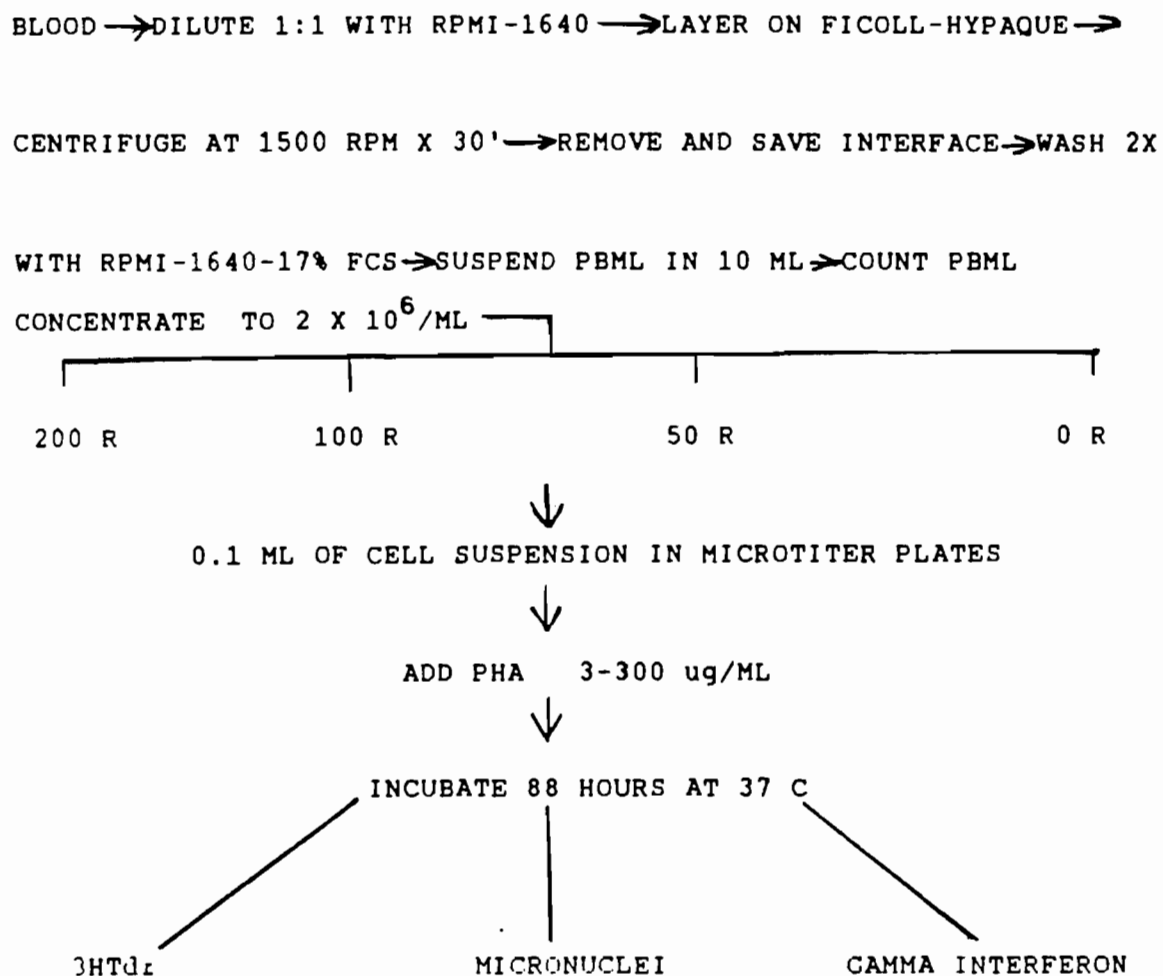


Fig. 1. Sequence of PBML isolation and general protocol.

DRAW HEPARINIZED BLOOD → SEPARATE PBML ON FICOLL -  
 HYPAQUE GRADIENT → CONCENTRATE PBML TO  $2 \times 10^6$ /ML  
 → IRRADIATE 4ML ALIQUOTS OF PBML → ADD 0.2 ML PBML  
 TO EACH WELL → ADD PHA DILUTIONS OF 3-300  $\mu$ G/ML  
 → INCUBATE AT 37°C FOR 88 HOURS

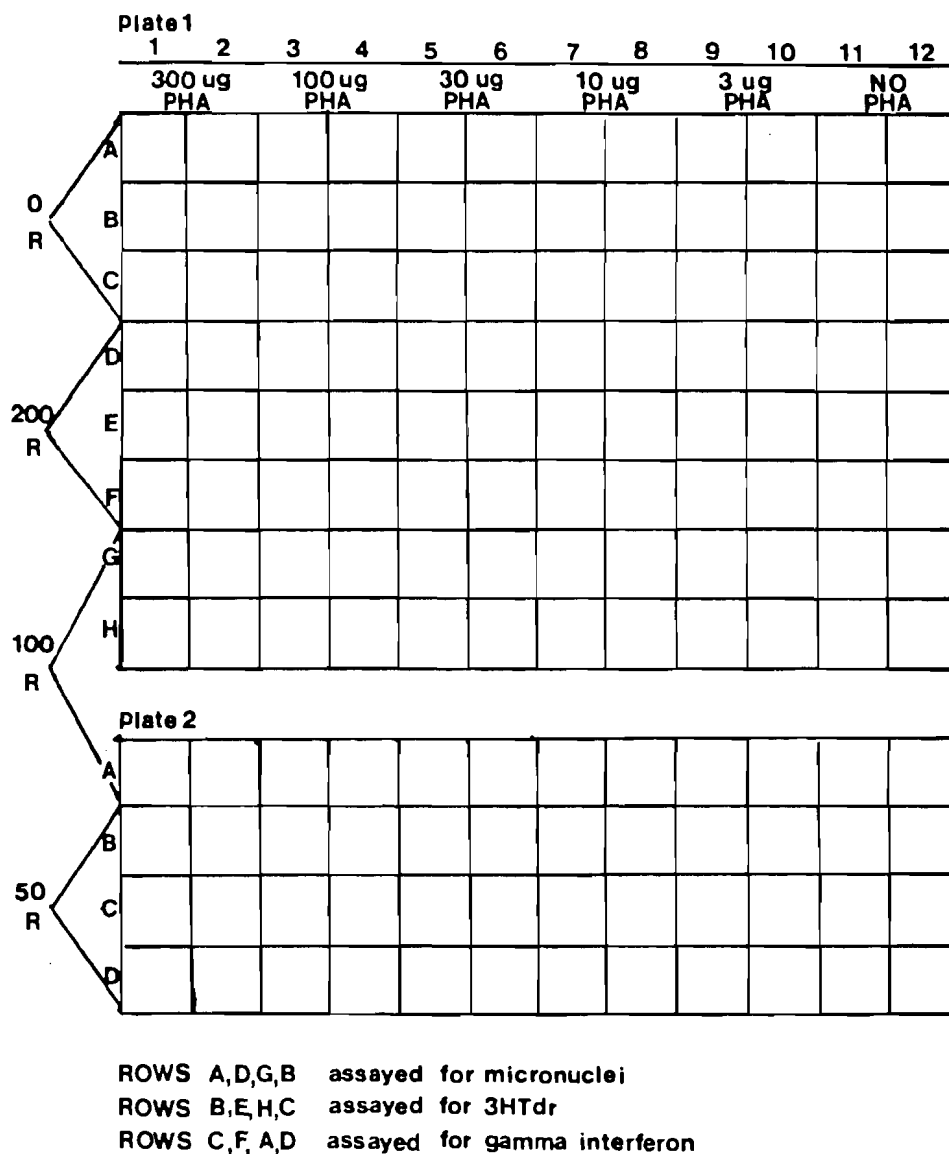


Figure 2. Sequence of PBML culture preparation (top) and schematic for PBML distribution in microculture plates for mn, 3HTdr, and gamma-IFN determinations (bottom).

received 200 rads; and the next 3 rows are cells that received 100 rads. The final 2 rows of the first microculture plate and the first row of the second plate contain cells that received 50 rads.

Each of the 3 horizontal rows is further divided so that the first row (row A) contains nonirradiated cells in duplicate with a complete PHA dose curve and cell control to be harvested for micronuclei at the end of the 88-hour incubation. The second row of the same subset (row B), also contains a complete PHA dose curve and cell control. However, 12 hours prior to harvest, 0.1 mCi/well 3HTdr (specific activity 20 mCi/mM) is added to the entire row of cells and returned to the incubator to complete the 88-hour incubation. Row C contains the same PHA dose curve, plus control, as in Rows A and B, but, following the incubation, the supernatants of these cultures are removed and frozen pending gamma interferon analysis. This protocol is repeated for the next 3 subsets of cells, which are exposed to the different amounts of gamma radiation. Figures 1 and 2 diagram the protocols for isolation of PBML and microculture.

The microculture plates are placed in a 5% CO<sub>2</sub>-humidified incubator for 88 hours. All samples are prepared in duplicate and, depending upon the protocol, in quadruplicate, with a final concentration of cells  $2 \times 10^5$ /well, and a final volume of 0.2 ml.

## 2.5 DNA Synthesis: Tritiated Thymidine Incorporation into Peripheral Blood Mononuclear Leukocyte DNA (Green, et al., 1981)

After a 12-hour incubation with 3HTdr, as described in Section 2.3, PBML DNA is isolated on glass fiber filters using a multiple automatic sample harvester (MASH, Flow Laboratories, McClean, Virginia). Filters are transferred to biogamma scintillation vials to which 4 ml of Aquasol counting solution is added. Tritiated thymidine incorporation into DNA is determined as disintegrations per minute in a Beckman Liquid Scintillation Counter.

## 2.6 Sample Preparation for Quantifying Micronuclei

Following an 88-hour incubation, the appropriate wells of cells are gently pipetted with individual Pasteur pipets. One drop of cell suspension is placed on a cytocentrifuge slide with 1 drop of glycerol and 2 drops of phosphate-buffered saline, pH 7.45. Cultures containing no or very low concentrations of PHA have 3 drops of cell suspension added to each slide. The increase in cell volume is necessary in cultures containing low concentrations of PHA. The entire slide is immediately spun in a table-top cytocentrifuge at 6500 rpm for 5 minutes.

Slides are Wrights-Giemsa stained by being immersed in methanol for 3 minutes, followed by immersion in stain

for 5 minutes and, finally, immersed in a buffer-stain solution for 15 minutes. The slides are gently washed under tap water, air-dried for 24 hours, and mounted with Permount. Staining times varied, depending upon the cell concentration and the batch of stain.

## 2.7 Quantifying Micronuclei--Modified

Stained slides are viewed with an oil immersion objective at a final magnification of 1000X to 1200X. The entire slide is scanned and 1000 cells are counted in randomly chosen, but representative, areas. The same pattern of scanning is utilized in all experiments.

Each slide is coded so the reader will not know the manner in which the PBML had been treated. Also, the micronuclei are differentially counted. This means that areas of the slide are selected regardless of whether they contain a micronucleated PBML. Once a field is randomly chosen, all cells in that field are quantitated, and the micronucleated cells are counted separately, but as part of the total number of cells. The numbers of micronuclei are expressed as a ratio out of 1000 total cells per slide.

The same criteria for counting micronuclei apply to all slides and to all experiments:

- a. The cell must be intact;



- b. The micronuclei must be completely separate from the main nucleus, but clearly within the cytoplasm;
- c. The micronuclei must be at least 50% smaller than the main nucleus;
- d. The reader may not preselect areas that contain micronuclei.

## 2.8 Gamma Interferon Assay (Yet, et al., 1984)

Following an 88-hour incubation, supernatant media of replicate cultures are removed with a Pasteur pipet. Media from duplicate cultures are pooled in 1 dram sterile, glass vials, labeled and frozen at  $-70^{\circ}\text{C}$  until assayed for activity. All gamma interferon determinations are made within 1 month from the time the supernatant has been removed.

WISH amnion cells are a continuous cell line of normal epithelial origin. They have a consistent morphology and a doubling time of approximately 24 hours. These cells are easily maintained as monolayers grown in RPMI-1640 containing 10% FBS. WISH cells can routinely be trypsinized from an adherent surface and be dispersed into single cells for ease of counting. The cells can then be subcultured in microculture wells and grown to confluency to serve as indicator cells for interferon activity.

Microculture plates are initially seeded with  $7 \times 10^4$  WISH cells/well in 0.1 ml volumes. The cells are allowed

to grow to confluency, which occurs in 24 hours. Samples to be tested for gamma interferon are added in 50  $\mu$ l volumes to the first row of each plate. Serial 1:3 dilutions are made with a Titer-Tek automatic dilutor (Flow Laboratories). Each plate consists of 8 horizontal rows and 12 columns. Row 11 of each plate is always a virus control, meaning that it contains only virus inoculum. Row 12 is always a cell control to which only media is added. After the dilutions are made, the plates are incubated at 37°C for a minimum of 6 hours to allow the gamma interferon to maximally activate the WISH cells to the antiviral state. Following the 6-hour incubation period, WISH cell culture media are decanted, and the cell monolayers are washed one time with 0.1 ml of prewarmed Eagle Minimal Essential Medium containing 2% FBS. Fresh medium containing encephalomyocarditis (EMC) virus is added at a ratio of 1 infectious particle per 10 WISH cells in 0.1 ml to all wells except the cell controls, which received 0.1 ml of virus-free medium. The titer of virus had been previously determined and was used by several laboratories. The plates are incubated for approximately 22 hours at 37°C, or when 95% of the WISH cells in the virus control wells show typical cytopathic effect (CPE) when viewed microscopically. Dramatic morphologic changes initiated by the virus include diffuse granulation, decrease in the size of the cells, appearance

of clusters on the monolayer, and loss of characteristic WISH cell morphology.

The medium is decanted, and the entire microculture plate is fixed and stained with crystal violet-formalin-ethanol-saline solution for 15 minutes. The plates are then washed under running tap water and air-dried. Microculture wells, which contain higher titers of interferon and are protected from the virus, stain a dark purple, while those cells with little or no interferon are colorless. Interferon titers are established by optical density reading of the plates using an automated ELISA reader (Multiscan, Flow Laboratories) equipped with a 600 nM filter. Interferon titer is expressed as the reciprocal of the dilution of supernatant that inhibits EMC-induced cytopathic effect (CPE) by 50%.

### 3. RESULTS

#### 3.1 Preliminary Experiments

A series of slides prepared for teaching purposes by Dr. Gordon Livingston was used to learn to identify micronuclei in PBML exposed to 100-400 R of gamma radiation and PHA. Duplicate slides were coded to permit impartial, e.g., "blinded," reading of 1000 PBML/slide. Dr. Livingston's techniques were used and microscopic fields containing micronucleated cells were selected exclusively. The method used to perform these preliminary experiments is described in Section 3.3. Figures 3 and 4 show micronuclei counts resulting from my reading the teaching slides compared with counts obtained with an experienced reader, respectively. The comparable nature of the results obtained, with small variations, by the two readers indicates that the investigator could count micronuclei accurately without further practice. At this point, prior to further experimentation, the method was altered to eliminate the potential bias that results from only selecting areas that contain PBML with micronuclei. Preselection of micronuclei containing microscopic fields was eliminated, and areas were chosen at random along a zig-zag pattern of the slide. Efforts were made to count PBML that were representative of the entire slide.

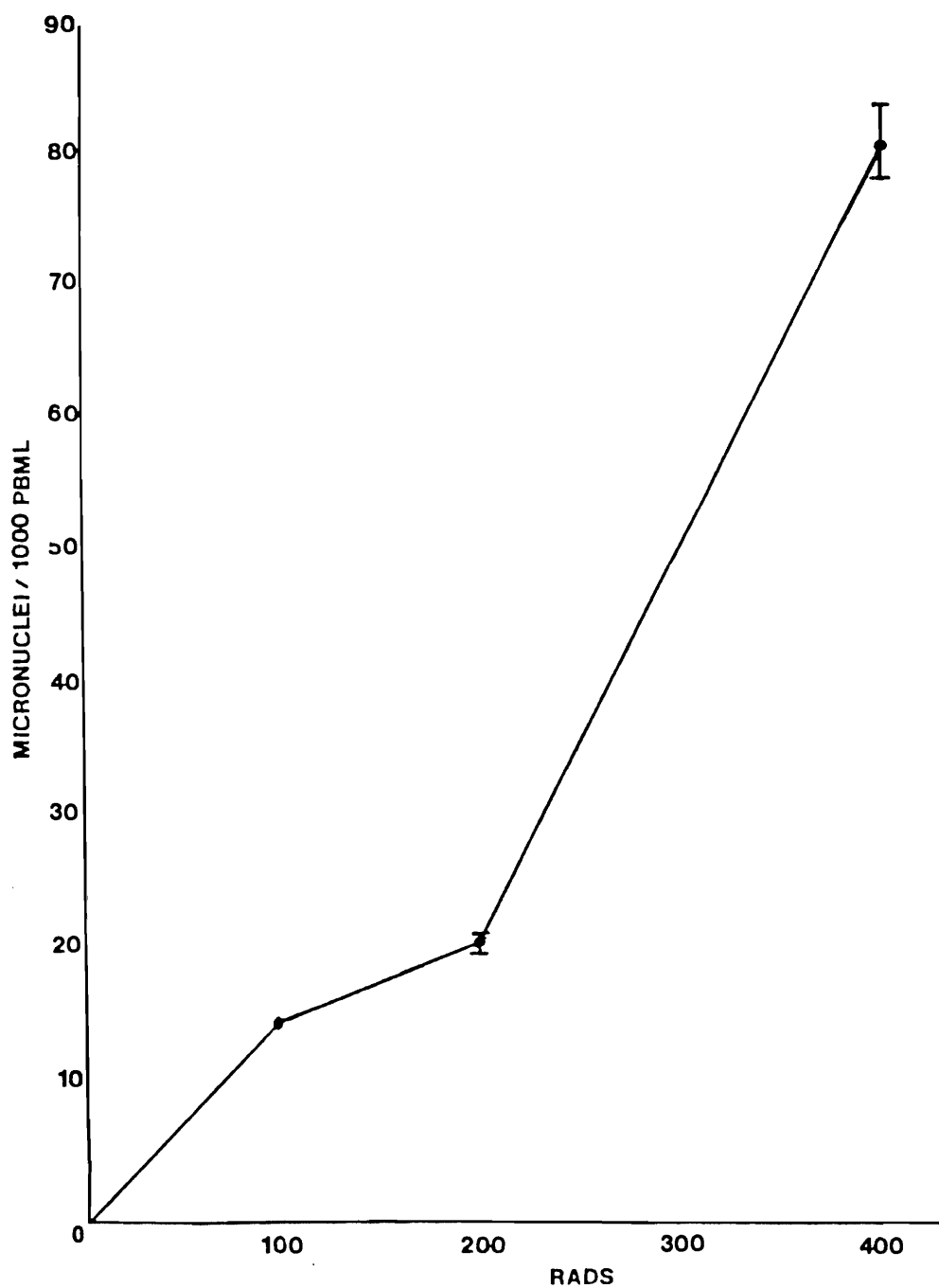


Figure 3. Micronuclei (mean  $\pm$  S.D.) counts from standard teaching slides read by investigator.

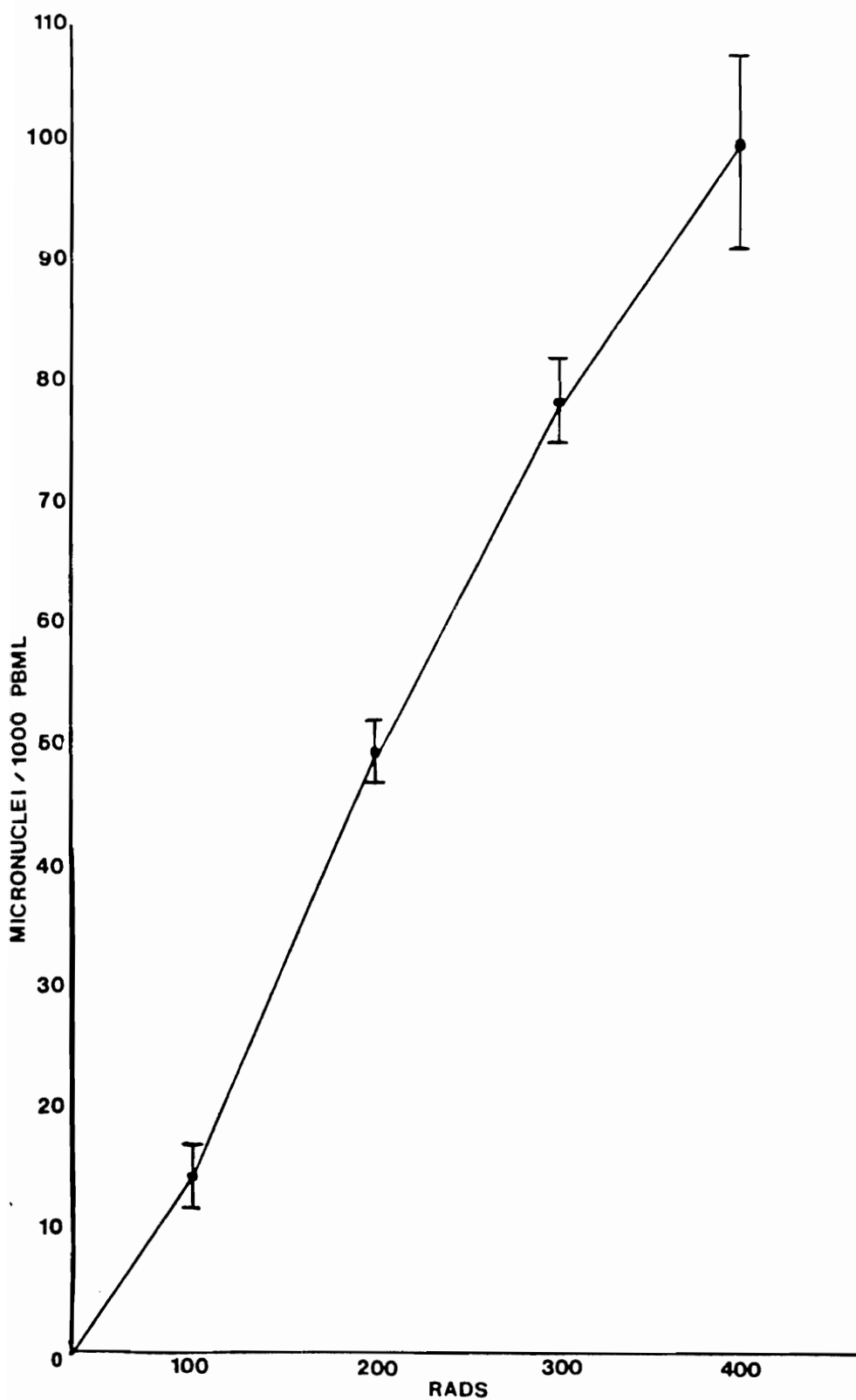


Figure 4. Micronuclei (mean  $\pm$  S.D.) present in PBML exposed to gamma radiation read by a trained reader (courtesy G. Livingston).

PBML not exposed to radiation did not produce micronuclei. Total numbers of micronuclei increased with the amount of radiation. Both readers observed peak micronuclei production at 400 R with a mean of 82.5/1000 lymphocytes in Figure 3 and 105/1000 lymphocytes in Figure 4. Micronuclei production increased in response to increased radiation with both readers. The teaching slides did not contain a non-PHA control, and the concentration of PHA added to the irradiated cells was not known.

Initial experiments did not yield results that can necessarily be expressed in numbers of micronuclei. Many methodological problems were incurred while trying to obtain micronuclei. These problems, and the resulting modifications to the existing assay, are as follows: First, duplicate cultures became a requisite part of each experiment. Second, non-PHA cultures of PBML were added as a control. Third, PBML in every experiment were adjusted to the same concentration of  $2 \times 10^6/\text{ml}$ . Fourth, a graded series of concentrations of PHA were added to fixed numbers of PBML following irradiation to see if micronuclei production was dependent on the concentration of mitogen as well as genotoxin. Fifth, numbers of PBML spun onto the cytocentrifuge slide needed to be increased in control cultures of PBML that did not contain PHA, as well as the cultures with lower

concentrations of PHA. Sixth, the large volume of the cultures (10 ml in a 15 ml conical centrifuge tube) created a potentially harmful pH gradient during incubation. A microculture system was adapted to the culture of PBML for micronuclei, which resulted in a final volume of 0.2 ml.

Figure 5 shows that PBML exposed to 200 R and concentrations of PHA that ranged from 25 ug/ml to 400 ug/ml produced micronuclei in consistently greater numbers than in the nonirradiated control culture containing 200 ug/ml PHA. At that concentration of PHA, the nonirradiated culture contained 7/1000 lymphocytes, while the irradiated counterpart contained 75/1000 lymphocytes. An apparent dose-related peak in micronuclei production was observed at 100 ug/ml PHA, with a mean of 134/1000 cells. The lowest concentration of PHA added to irradiated cells was 25 ug/ml, and the 65/1000 lymphocytes counted at this concentration was approximately 9 times higher than that found in the nonirradiated control.

Examination of the cultures following the 88-hour incubation period revealed that the pH of the cultures, estimated by variation in color of the phenol red containing medium, decreased as the concentration of PHA increased. The tubes containing the control and low PHA concentrations were red-purple, while the tubes



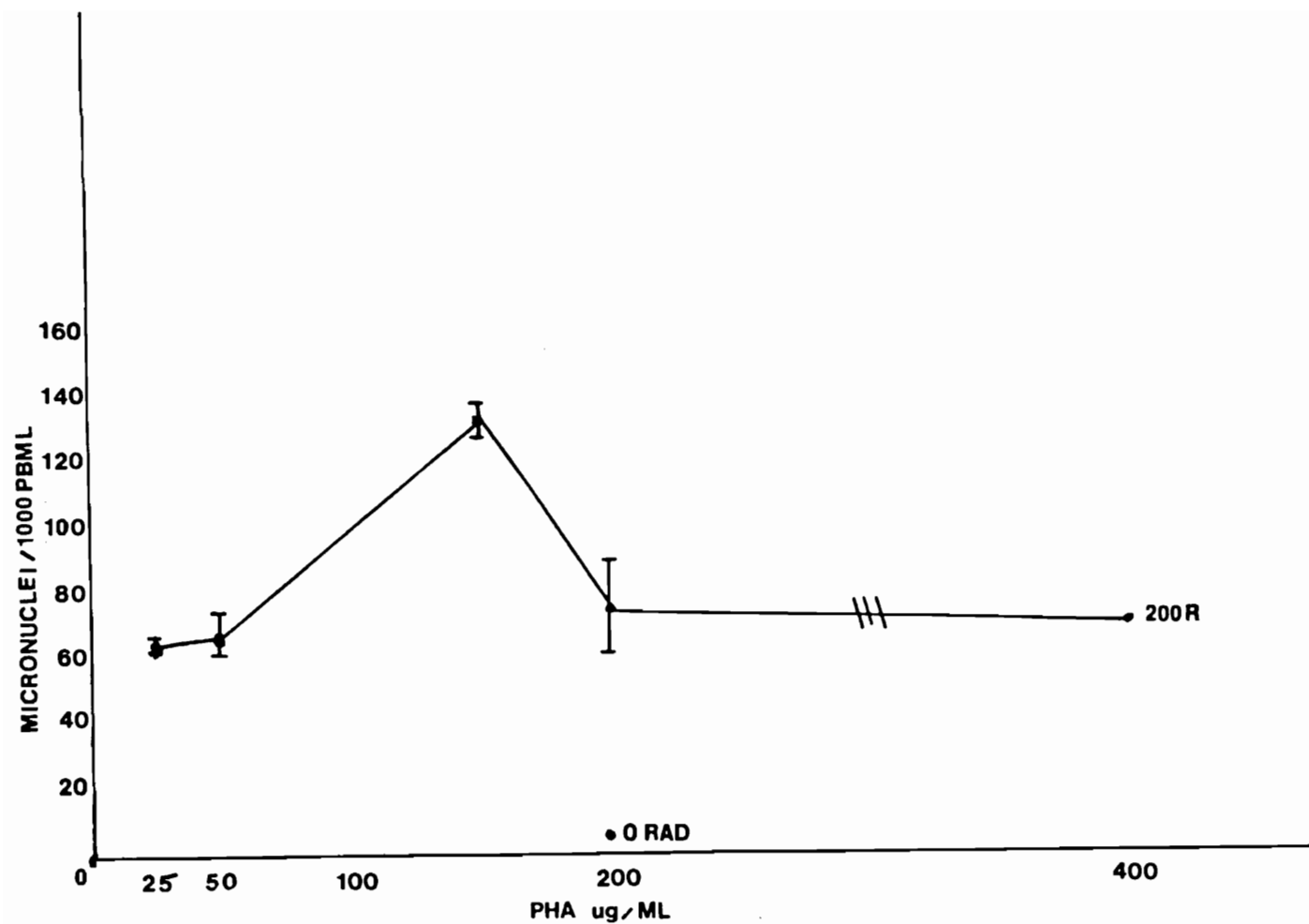


Figure 5. Micronuclei (mean  $\pm$  S.D.) in PBML incubated with PHA (25-400 ug/ml) following exposure to 200 R of gamma radiation.

containing the 200 ug/ml and 400 ug/ml PHA were yellow. Several slides were not able to be read because of massive blast transformation and cell fragmentation that were noticed when PBML were exposed to high concentrations of PHA and 200 R of gamma radiation. Table 1 presents the previously used method and the modified methods that were used in the next experiments. Optimum culture conditions were considered to be vitally important to this method and it was clear, following microscopic examination of the cells, that large volumes were not ideal. Modifications listed above, and those described in Section 2.7, were used in the remaining experiments. These modifications provided the prerequisite standardization necessary for the study of the induction of micronuclei.

### 3.2 Micronuclei Production in Gamma-Irradiated Peripheral Blood Lymphocytes Performed in Microculture

The relationship among radiation exposure, PHA concentration, and micronuclei production was examined in PBML maintained in microcultures in multiple-well plates. Replicate cultures were prepared with PBML that were either exposed to 200 R or were nonirradiated. Irradiated and nonirradiated PBML were cultured with PHA concentrations ranging from 25 ug/ml to 400 ug/ml.

TABLE 1  
COMPARISON OF PREMODIFICATION AND MODIFIED  
MICRONUCLEUS ASSAYS

Premodification Method	Modified Method
PBML concentration unknown	PBML concentration fixed
Culture volume 5-15 ml	Culture volume 0.2 ml
Select only micronuclei containing PBML fields	Random fields of PBML chosen
No non-PHA controls	Non-PHA controls in each experiment
One concentration of PHA	Range of PHA concentrations
PBML incubated in closed tubes in 37°C incubator	PBML incubated in 5% CO humidified incubator at 37°C

The number of micronuclei in irradiated and nonirradiated cells is presented in Figure 6. There were no micronuclei in cultures without PHA or radiation. A mean of 5.5/1000 lymphocytes was obtained in the 175 ug/ml of PHA cultures. The remainder of the nonirradiated cultures produced no greater than 3/1000 lymphocytes, regardless of increasing concentrations of PHA. Microscopic examination of the nonirradiated PBML revealed cells in varying stages of blast transformation that increased in number as PHA concentration increased. Small lymphocytes were prominent in the cultures PBML containing low concentrations of PHA.

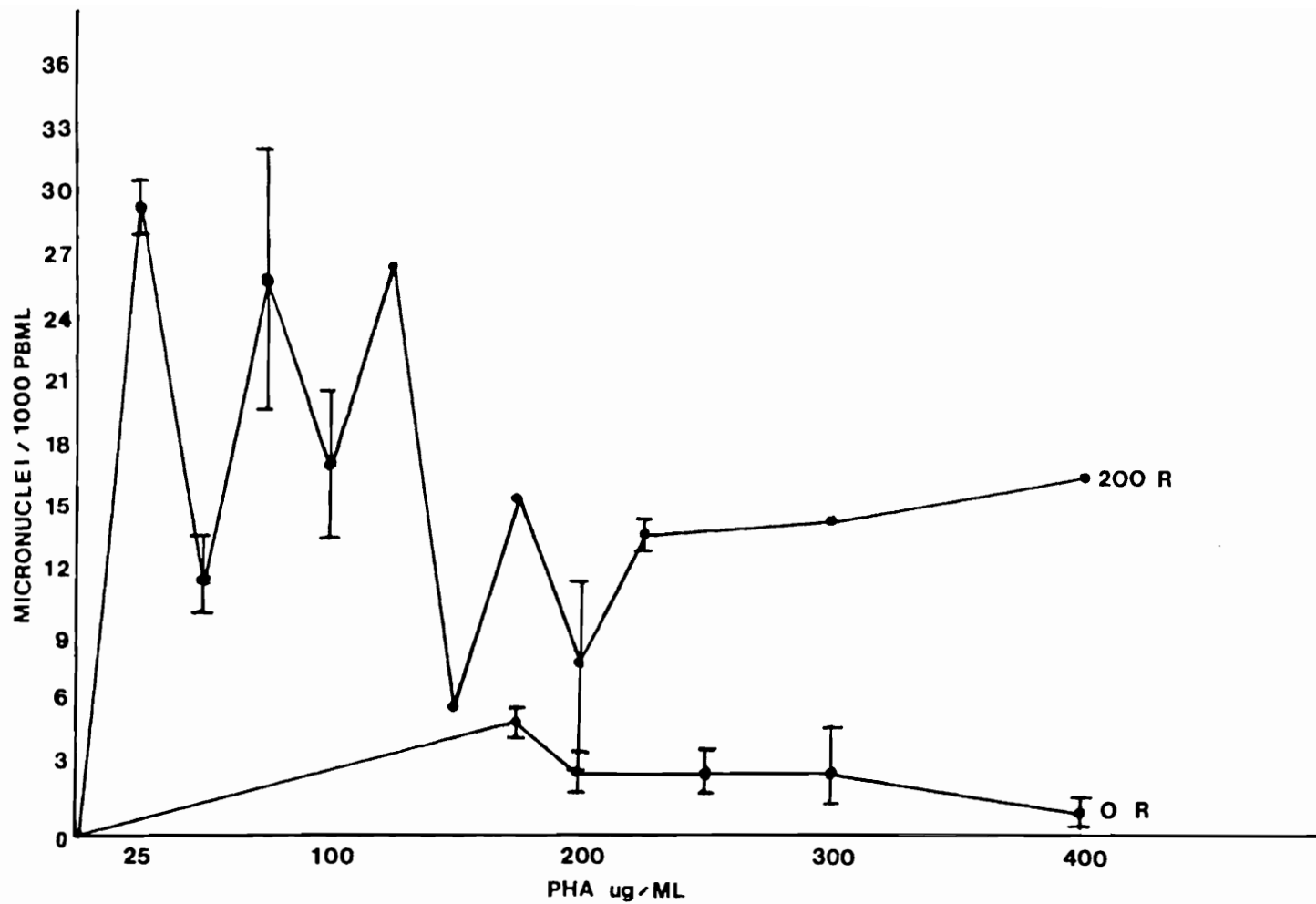


Figure 6. Micronuclei (mean  $\pm$  S.D.) in PBML exposed to 200 R of gamma radiation and incubated in medium with and without PHA.

PBML that were irradiated and exposed to varying concentrations of PHA produced micronuclei. Irradiated cultures to which PHA was not added exhibited extensive cell damage, and no micronuclei were found. Stained preparations of these cultures contained almost 100% small lymphocytes, which were either fragmented, with their cytoplasm extruding from the cell, or appeared much smaller and more condensed than normal, with no visible cytoplasm, or were lysed. Radiation-induced cell damage was not seen in slides prepared from cultures that also contained PHA. Cultures containing the lowest concentration of PHA, 25 ug/ml, induced a mean of 29.5/1000 lymphocytes, and the PBML on these slides appeared uniformly healthy. There were many small lymphocytes with visible cytoplasm scattered among blast cells, as well as small clumps of agglutinated blast cells.

Micronuclei were visible only in the blast transformed cells. Concentrations of PHA in excess of 100 ug/ml produced variable, but decreasing, numbers of micronuclei. In this initial microculture experiment, there were wide ranges in micronuclei counts and poor duplication. It appears that peak micronuclei production occurred between 25 ug/ml and 125 ug/ml PHA. It was not clear, at this point, why the curve seemed to follow a downward trend with higher concentrations of PHA. Increasingly large numbers of blast-transformed cells and

agglutination were observed with higher concentrations of PHA, and, yet, mean micronuclei counts decreased to 17.5/1000 lymphocytes at 400 ug/ml PHA. This experiment was repeated in an attempt to achieve better duplication and to examine the inverse relationship between increasing PHA concentration and decreased in micronuclei production.

Another experiment (Experiment A), which is presented in Figure 7, shows more clearly that micronuclei production was greatest at the lowest concentration of PHA and declined. Slide preparations appeared uniform in cell number, and accuracy in duplication increased with corrected cell volumes and uniformity of stain. Control cultures without irradiation and PHA contained no micronuclei. Numbers of micronuclei in nonirradiated PBML, regardless of PHA concentration, averaged 3.1/1000 lymphocytes with a range of 1-5/1000 lymphocytes. Maximum numbers of micronuclei, 53/1000 lymphocytes, were found in irradiated cultures containing 75 ug/ml of PHA. Absolute numbers of micronuclei decreased to a mean of 15.5/1000 lymphocytes in the cultures containing 400 ug/ml PHA. Microscopic examination of irradiated cells at the higher concentrations of PHA revealed varying degrees of cell damage. PBML were considered damaged if any or all of the following were observed: broken cell membranes, extruded cytoplasm, massive enlargement of the

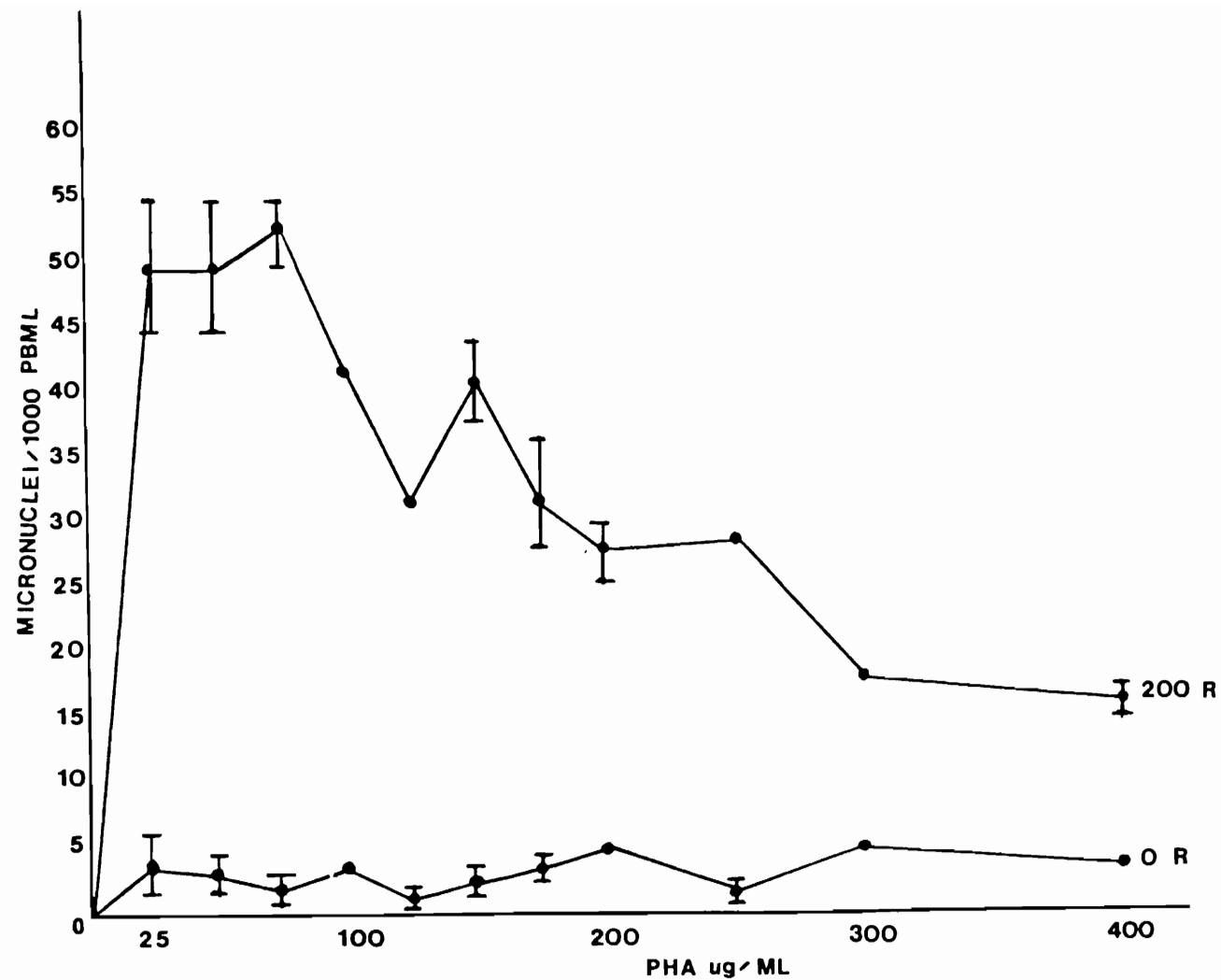


Fig. 7. Experiment A: Micronuclei (mean  $\pm$  S.D.) in PBML exposed to 200 R of gamma radiation and incubated in medium with and without PHA.

cell with lacy cytoplasm, torn nuclear membranes, or pyknotic appearance.

### 3.3 Micronuclei Formation, DNA Synthesis, and Gamma Interferon Production in Irradiated and Nonirradiated Peripheral Blood Lymphocytes

The effect of several doses of radiation combined with lower concentrations of PHA on micronuclei production is shown in Figure 8 (Experiment B). There were no micronuclei in PBML from control cultures without radiation or PHA. Nonirradiated cultures contained no or very few micronuclei, with a mean of 1/1000 lymphocytes at 100 ug/ml of PHA, and a mean of 2/1000 lymphocytes at 300 ug/ml of PHA. Microscopic evaluation revealed evenly stained, small lymphocytes with only scant blast transformation. There was no agglutination at low concentrations of PHA, but, as noted in previous experiments, blast transformation and agglutination increased with PHA concentration.

Irradiated PBML began to produce micronuclei at 10 ug/ml of PHA regardless of dose of radiation, but the higher doses of radiation produced greater numbers of micronuclei. In the 50 R cultures, maximum micronuclei production with a mean of 17.5/1000 lymphocytes at 30 ug/ml of PHA was reached. The same number of micronuclei were present at 100 ug/ml of PHA, then dropped to 4/1000 lymphocytes at 300 ug/ml of PHA. Micronuclei production



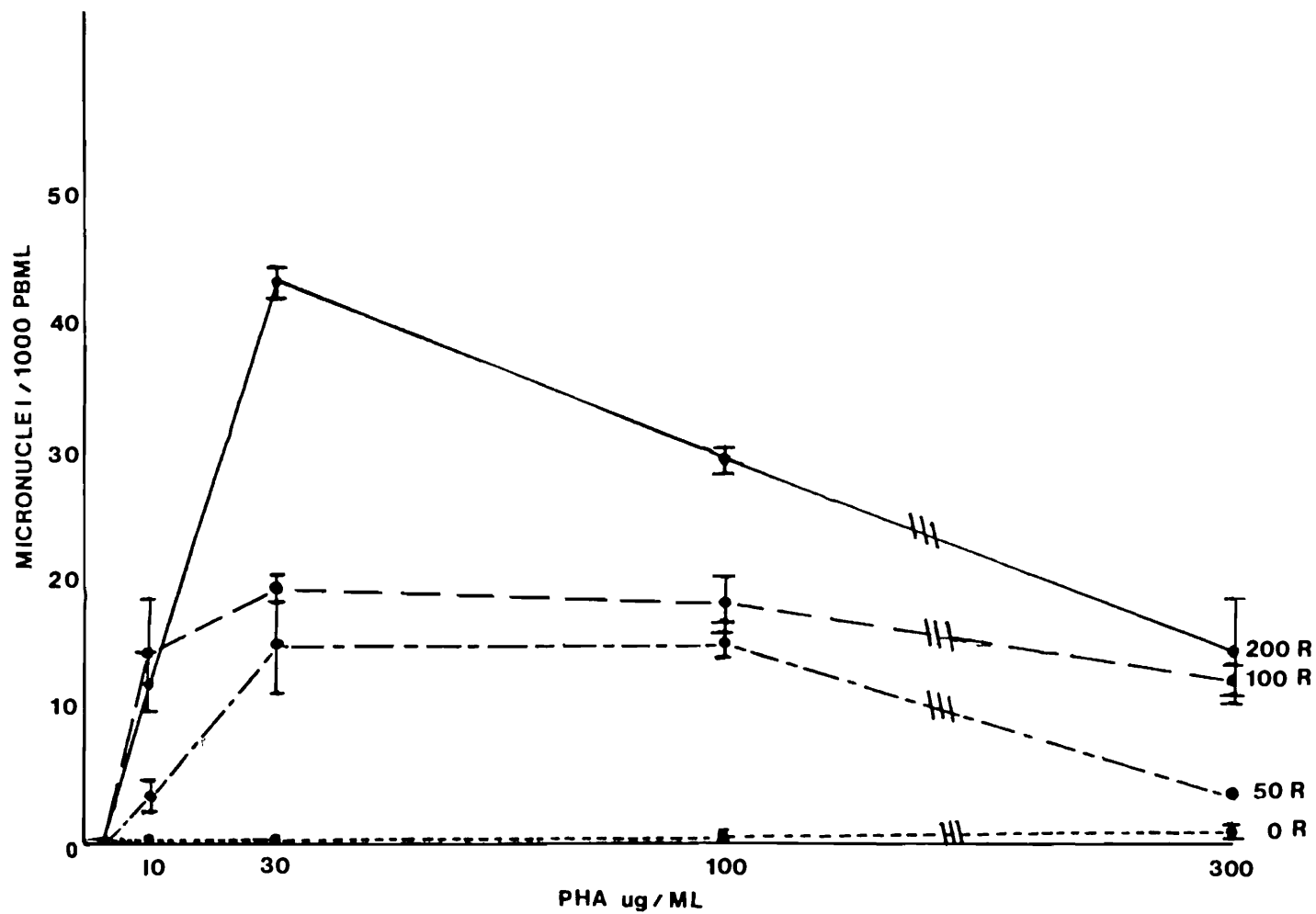


Fig. 8. Experiment B: Micronuclei (mean  $\pm$  S.D.) in PBML exposed to gamma radiation (50-200 R) and incubated in medium with and without PHA.

was similar in the 100 R and 50 R cultures. The greatest number of micronuclei were produced at 200 R and 30 ug/ml of PHA, with a mean of 44/1000 lymphocytes. The number of micronuclei declined to 30/1000 lymphocytes with 100 ug/ml of PHA, and, at 300 ug/ml of PHA, were within the range of response elicited at 10 ug/ml of PHA.

Cell morphology changed with increasing concentration of PHA and increasing radiation. Control cultures without PHA contained 99% small lymphocytes. Morphologic integrity of the control cultures decreased as the radiation exposure increased. The control cultures at 200 R contained more pyknotic small lymphocytes, and some cell debris was evident. There was no debris at the lower doses of radiation. Visual examination suggested that cell number increased in cultures that contained higher PHA concentrations. Lymphocytes exposed to PHA concentrations of 30 ug/ml or higher were 99% blast transformed. Many cells were observed in various stages of mitosis. Some cells, in which the spindle had already formed, had poorly defined and densely stained material that appeared to be lagging near the metaphase plate after the chromosomes were almost at the centromere. The fate of this nonspindle associated chromosomal material has not been determined. Cells observed in later stages of mitosis continued to reveal these areas, which appeared to become more dense.

PBML exposed to the highest radiation doses and cultured with the highest concentrations of PHA were larger than commonly observed blast cells, and some had disrupted membranes. The cytoplasm of these cells had a lacy appearance and stained a very pale blue. Agglutination was so extensive that individual cell morphology was difficult to distinguish. Micronuclei contained in these very large blast-transformed cells were also larger than the micronuclei seen in cultures with lower concentrations of PHA and irradiation.

Figure 9 (Experiment B) is a graph representing tritiated thymidine (3HTdr) uptake in PBML cultured concomitantly with PBML treated for micronuclei production. These data indicate that DNA synthesis is not impaired or enhanced by radiation. Control cultures without PHA or radiation produced 666 cpm. Nonirradiated cells showed an increase in uptake of 3HTdr as counts rose to 1911 cpm at 3 ug/ml of PHA, 33,320 cpm at 10 ug/ml of PHA, and peaking at 201,635 cpm at 300 ug/ml of PHA.

Maximum DNA synthesis and micronuclei production were achieved at 30 ug/ml of PHA, regardless of the dose of radiation. However, DNA synthesis is not affected by radiation, as can be seen in Figure 9. The decline in numbers of micronuclei in irradiated PBML at higher than 30 ug/ml of PHA was not reproduced in tritiated thymidine

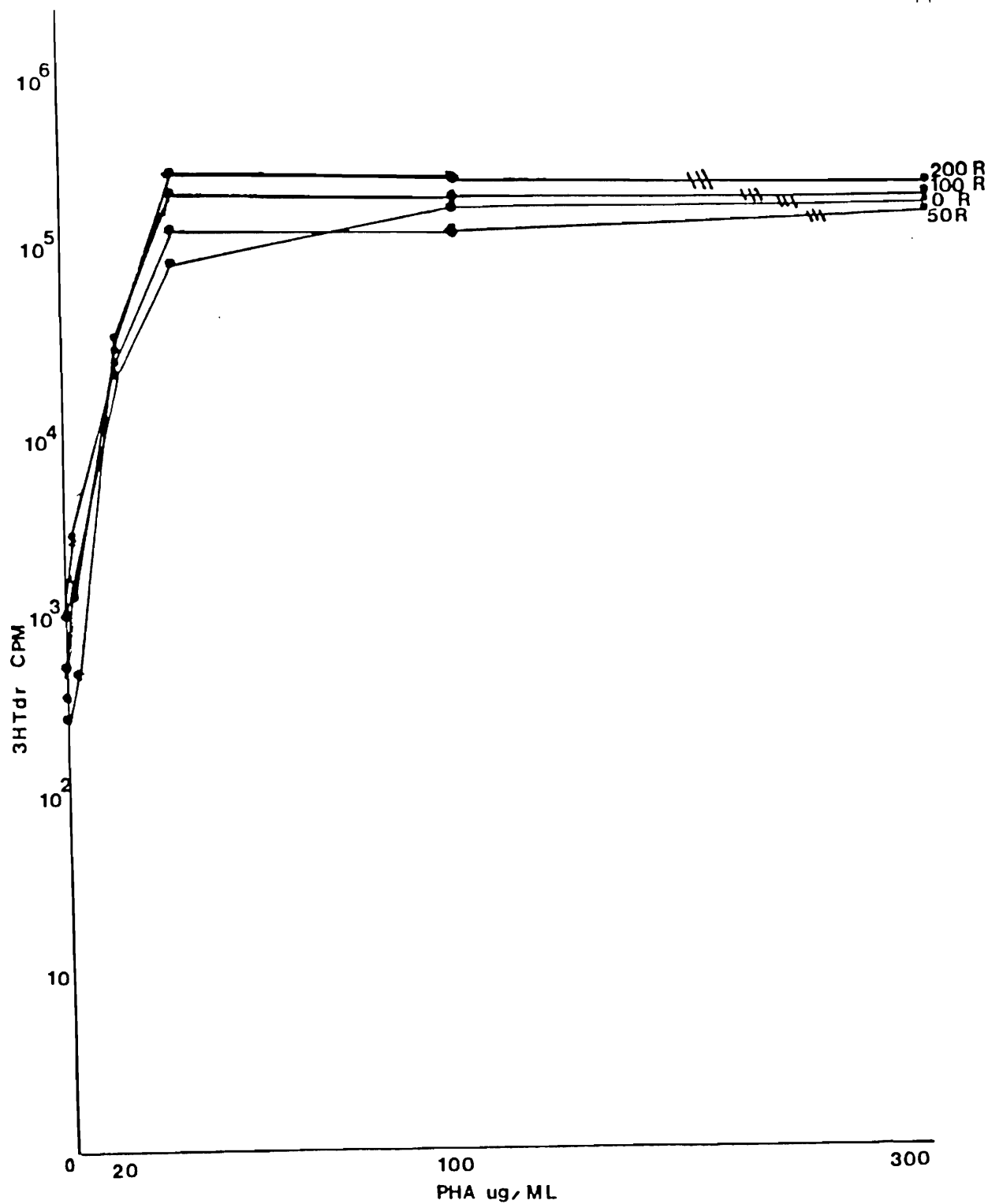


Figure 9. Experiment B: DNA synthesis--3HTdr incorporation into DNA by PBML incubated with a range of concentrations of PHA following exposure to gamma radiation (50-200 R).

uptake, indicating that it is unlikely that decreasing numbers of micronuclei are due to cell death.

Interferon titers varied independently from micronuclei formation and tritiated thymidine into DNA. Figure 10 (Experiment B) shows there were less than 3 units of interferon produced with no PHA or with 3 ug/ml of PHA. Interferon production was variable at 30 ug/ml of PHA, a concentration where maximum micronuclei were consistently produced. The highest titer of interferon was induced by 100 R, which produced 460 units of gamma interferon. Almost 100 units were induced in the non-irradiated cultures. PBML exposed to 50 R produced increasingly higher titers of gamma interferon as PHA concentration increased to 300 ug/ml of PHA. The latter concentration of PHA induced the production of 550 units of gamma interferon.

The data obtained in a subsequent experiment (Figure 11, Experiment C) were consistent with the previous experiment in that peak micronuclei production was reached at 30 ug/ml of PHA and 200 R. The control cultures without PHA or radiation did not contain micronuclei. A mean of 1/1000 lymphocytes was counted in the nonirradiated cultures containing 10 ug/ml, 30 ug/ml, and 100 ug/ml of PHA. The 300 ug/ml of PHA cultures contained 5.5/1000 lymphocytes. As seen in the previous experiments described in this paper, there continued to

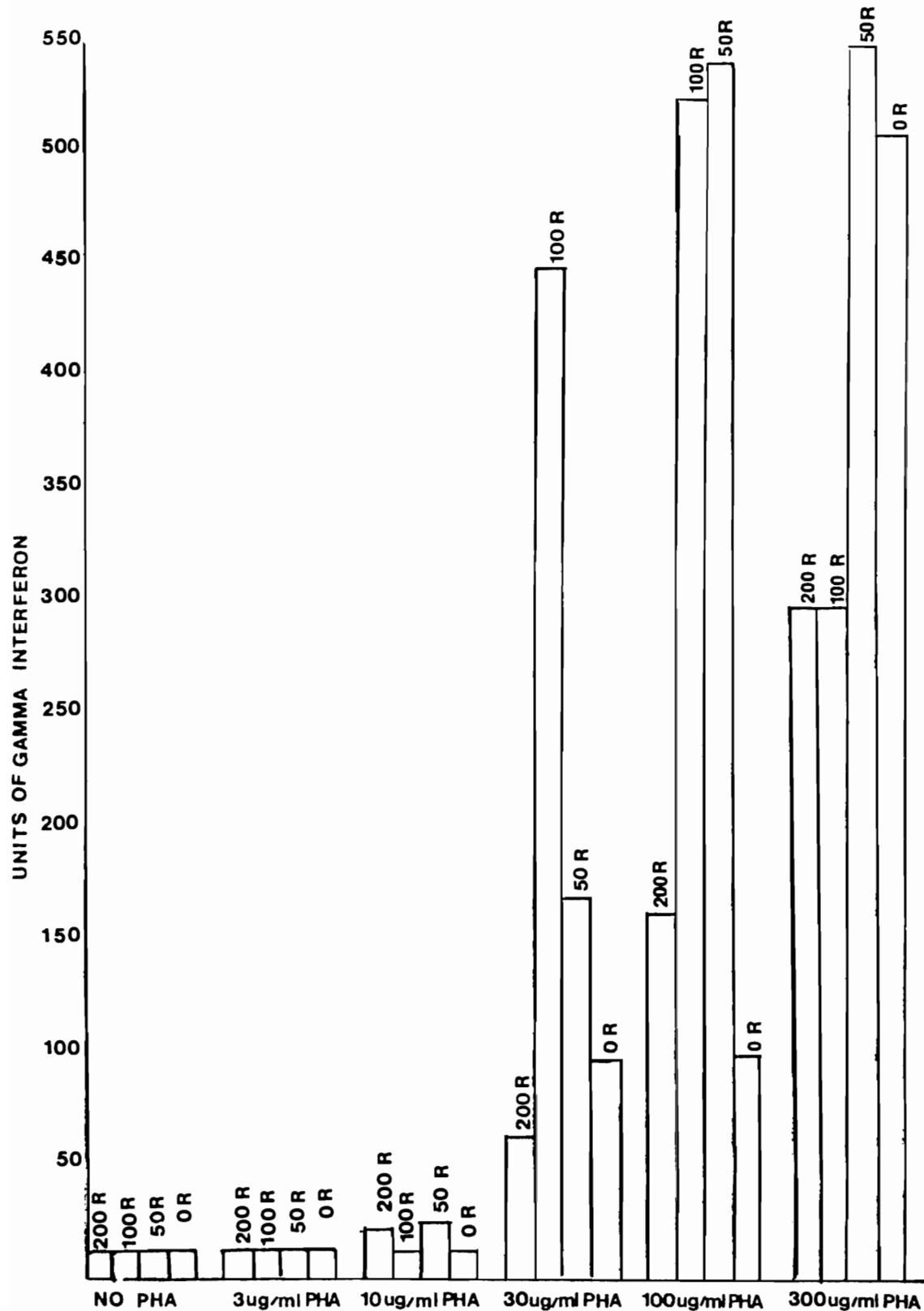


Fig. 10. Gamma interferon production by PBML incubated with a range of concentrations of PHA following exposure to gamma radiation (50-200 R).

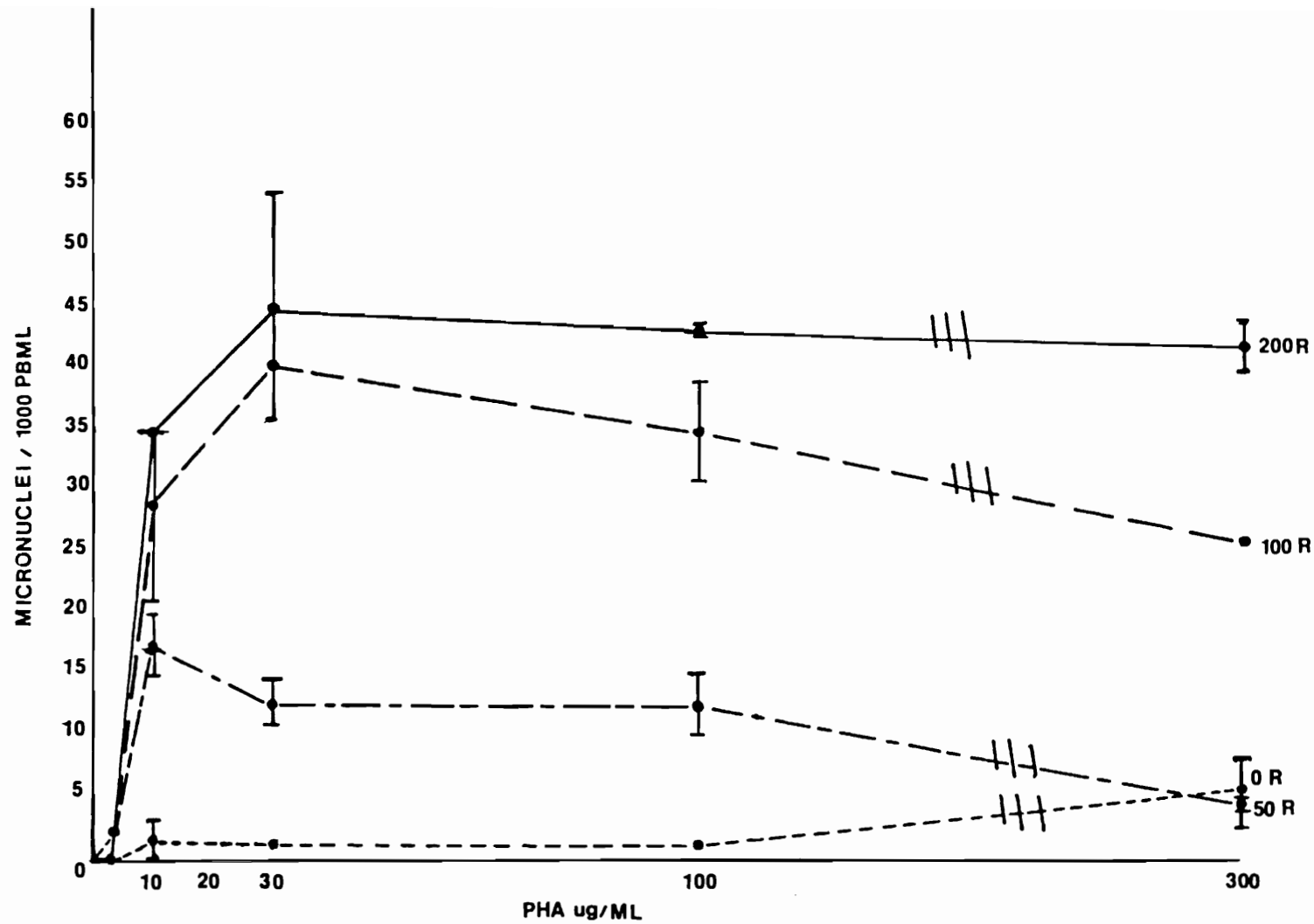


Fig. 11. Experiment C: Micronuclei (mean  $\pm$  S.D.) in PBML incubated with a range of PHA following exposure to gamma radiation (50-200 R).

be no dose-response curve to PHA without radiation. A mean of 47/1000 lymphocytes was counted in the cultures containing 30 ug/ml of PHA and 200 R. All control cultures without PHA, regardless of radiation levels, contained no micronuclei.

Tritiated thymidine incorporation into DNA increased with PHA concentration. These data are presented graphically in Figure 12 (Experiment C) and summarized in Table 2. The intensity of uptake of 3HTdr was similar to the prior experiment and was unaffected by radiation. Micronuclei production in the 200 R cultures did not decline as rapidly in this experiment; however, the number of micronuclei was less at 300 ug/ml of PHA.

Interferon titers could not be determined in this experiment because of toxicity of the microculture plates. Toxicity in the plastic interfered with the growth of the WISH amnion monolayer. However, preliminary results indicated there were less than 3 units of interferon produced at all radiation exposures in the controls, 3 ug/ml, and 10 ug/ml of PHA. The higher concentrations of PHA induced greater than 100 units of interferon, but the results were highly variable, due to the toxicity.

Results obtained in prior experiments were confirmed in an additional experiment, which assayed blood simultaneously from 2 donors. Protocols for this experiment,



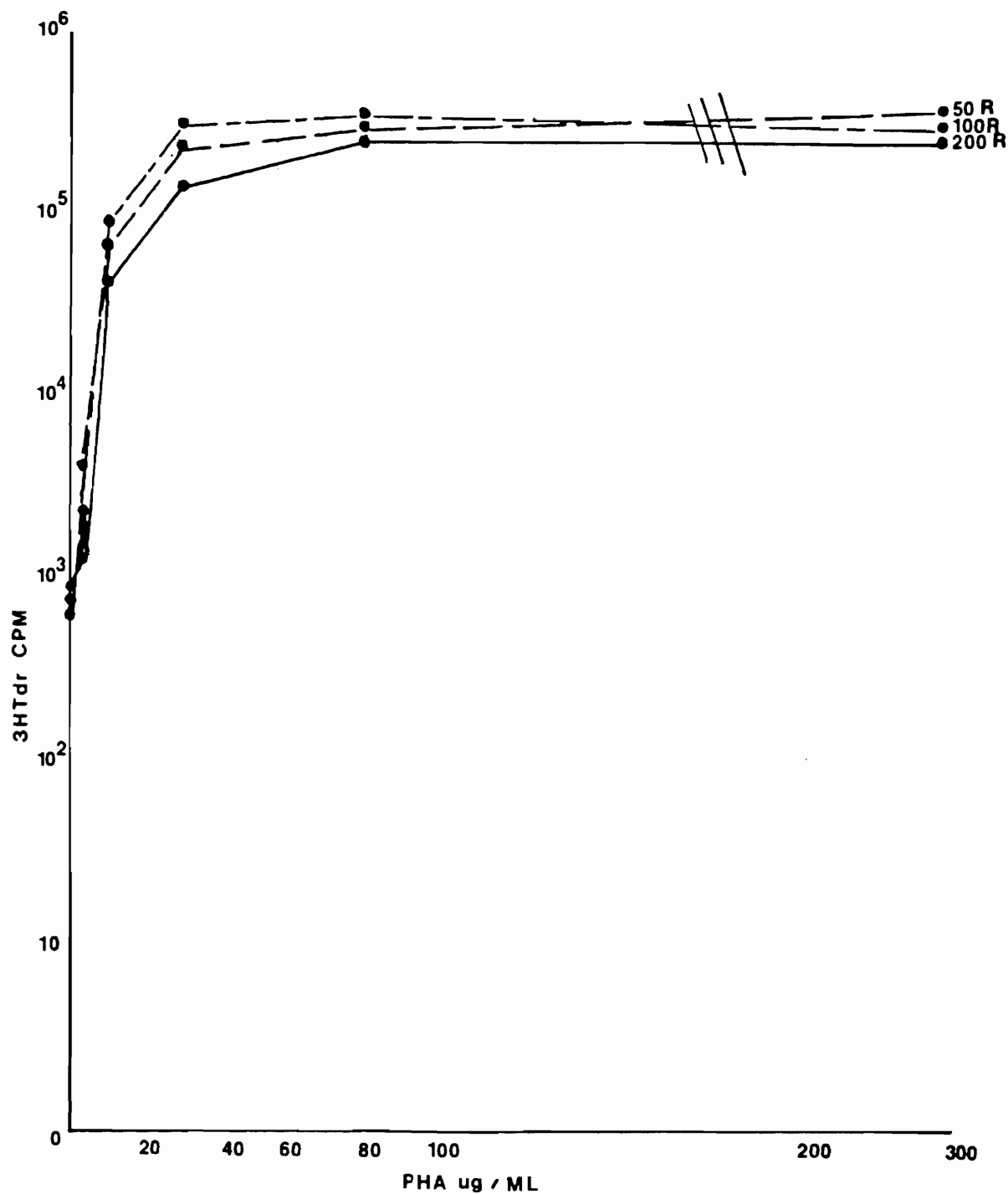


Figure 12. Experiment C: DNA synthesis--3HTdr incorporation into DNA of PBML incubated with a range of concentrations of PHA following exposure to gamma radiation (50-200 R).

TABLE 2  
EXPERIMENTS B AND C: 3HTdr INCORPORATION  
INTO DNA OF LYMPHOCYTES (CPM)

	PHA	Radiation (Rads)			
	(ug/ml)	0	50	100	200
<u>Experiment B</u>					
	300	201635	147090	166374	167902
	100	161018	142530	161574	204483
	30	181164	103624	199441	235853
	10	33320	103624	199441	235853
	3	1911	3807	667	1997
	0	666	141	307	427
<u>Experiment C</u>					
	300	N.A.	297775	237761	259030
	100	N.A.	247180	263330	199998
	30	N.A.	186057	220816	160995
	10	N.A.	76834	98107	48184
	3	N.A.	1258	2475	4595
	0	N.A.	969	853	771

as well as the 2 preceding experiments, are described in Sections 2.2 and 2.3. Figures 13 (Experiment D) and 14 (Experiment E) are graphs of micronuclei production by PBML from the individual blood donors. Figures 15 (Experiment D) and 16 (Experiment E) show the 3HTdr results obtained from the replicate cultures. Interferon data were not plotted because of continued toxicity problems and inconsistent data.

There were no micronuclei in the nonPHA, nonirradiated controls from Experiment D. There were also no micronuclei in all PHA controls. The nonirradiated cultures contained no micronuclei until a concentration of 30 ug/ml of PHA was reached, with a mean of 2.5/1000 lymphocytes. Peak micronuclei production occurred with 30 ug/ml of PHA and 200 R, where a mean of 55/1000 lymphocytes was produced.

Data obtained with PBML from Experiment E were similar to those for Experiment D and previous experiments. Similarities included no micronuclei in the control cultures and peak micronuclei production at 30 ug/ml of PHA and 200 R, where a mean of 80/1000 lymphocytes were produced. Notes taken at the time that the code was broken indicated a similar observation as in Experiment D, that the blast transformed cells in the high radiation cultures appeared to be healthy, while the small lymphocytes were fragmented and smaller than usual.

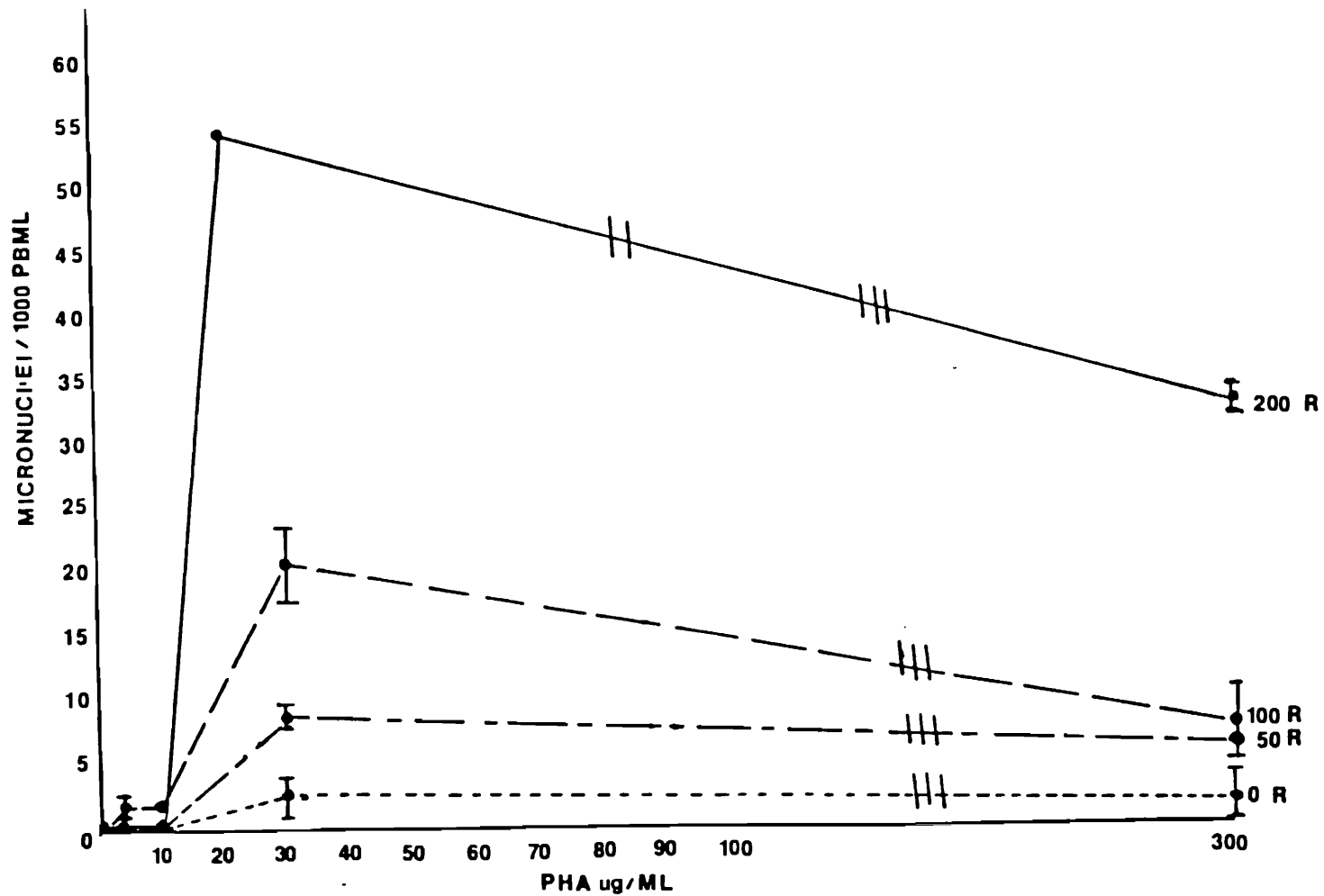


Figure 13. Experiment D: Micronuclei (mean  $\pm$  S.D.) in PBML exposed to gamma radiation (50-200 R) and incubated in medium with and without PHA.

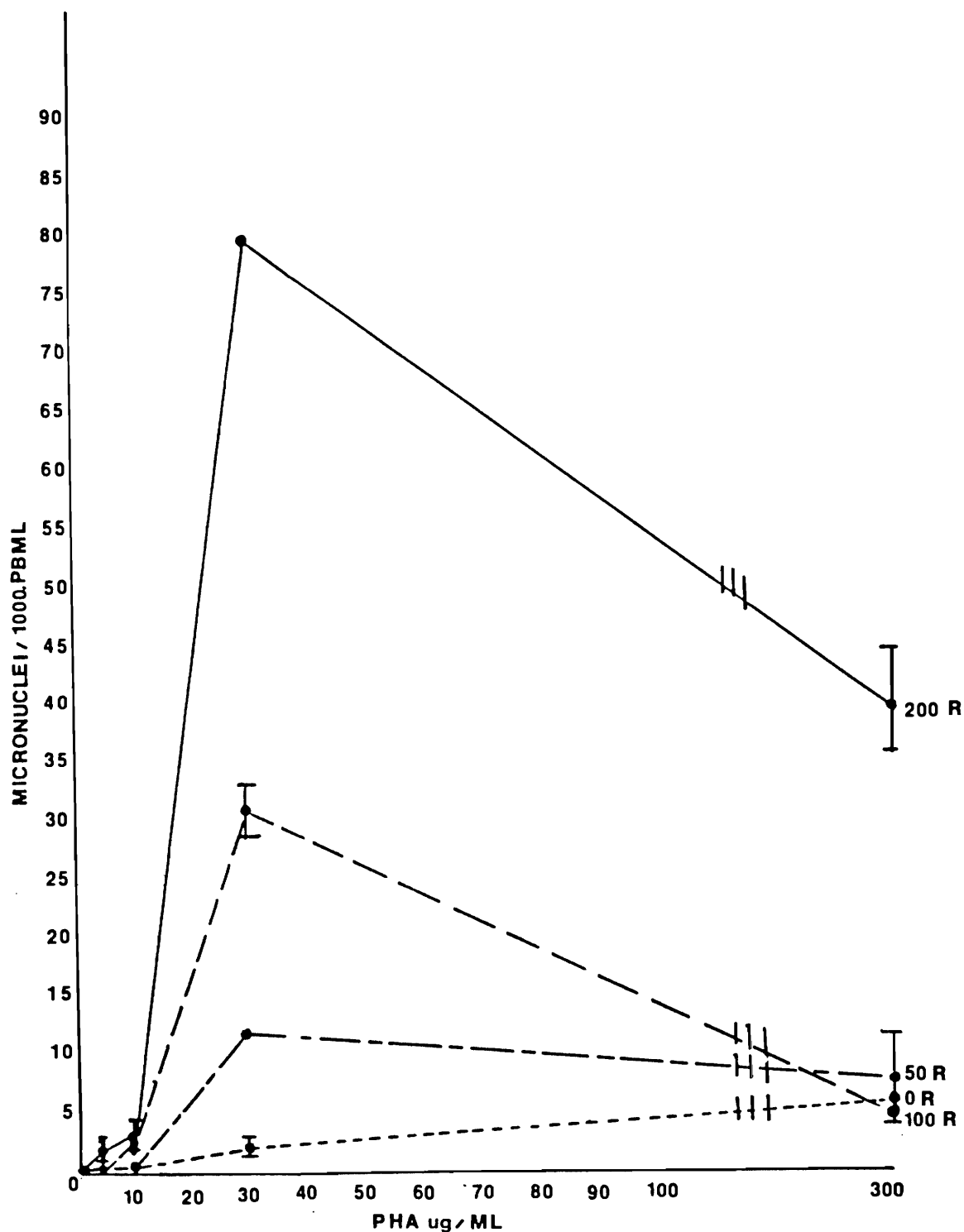


Fig. 14. Experiment E: Micronuclei (mean  $\pm$  S.D.) in PBML exposed to gamma radiation (50-200 R) and incubated in medium with and without PHA.

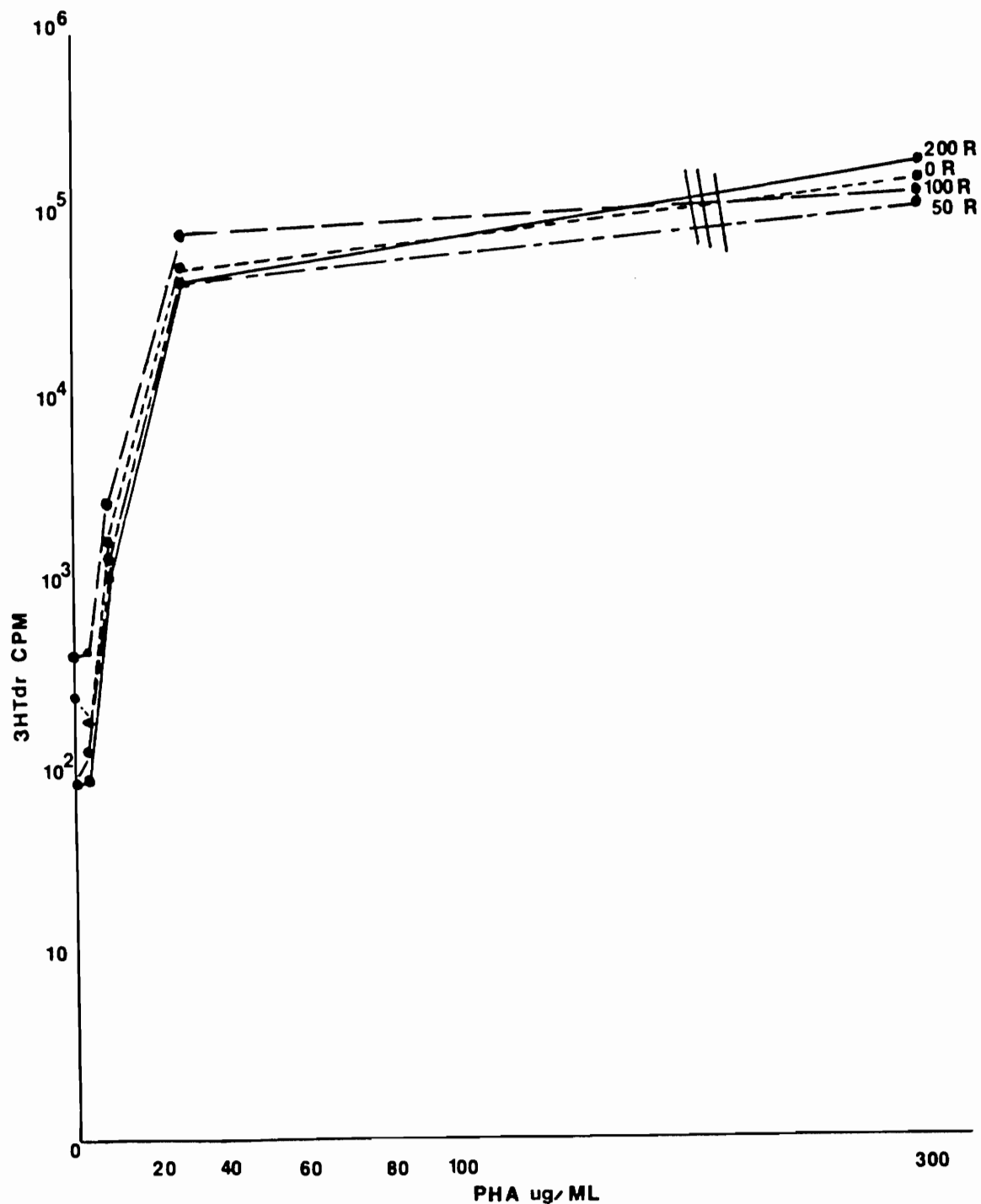


Figure 15. Experiment D: DNA synthesis--3HTdr incorporation into DNA of PBML incubated with a range of concentrations of PHA following exposure to gamma radiation (50-200 R).

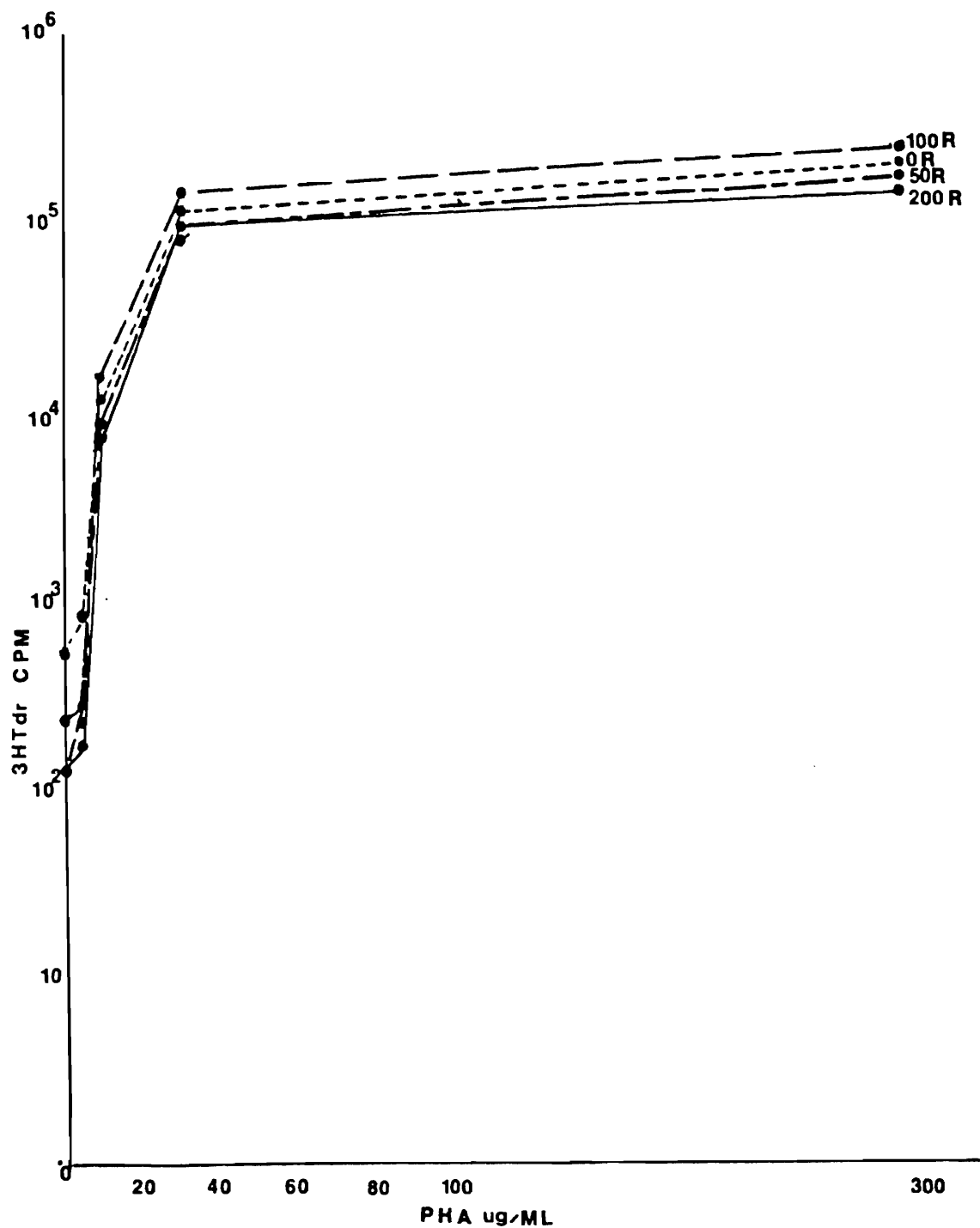


Figure 16. Experiment E: DNA synthesis--3HTdr incorporation into DNA of PBML incubated with a range of concentrations of PHA following exposure to gamma radiation (50-200 R).

It was also noted that the extreme blast transformation and agglutination in these high PHA concentration cultures made it difficult to identify micronuclei. It was necessary to look at the periphery of large, dense clumps of cells that contained more cells than could be counted.

Tritiated thymidine uptake into DNA in Experiments D and E was similar to the two previous experiments in that radiation did not inhibit DNA synthesis. Uptake of 3HTdr increased with concentration of PHA and remained consistently high. These data are presented in Table 3.

TABLE 3  
EXPERIMENTS D AND E: 3HTdr INCORPORATION  
INTO DNA OF LYMPHOCYTES (CPM)

		Radiation (Rads)			
PHA (ug/ml)		0	50	100	200
<u>Experiment D</u>					
300	170180	186050	160400	146974	
	30	51117	40403	83606	44525
	10	1684	1696	2649	1242
	3	188	421	84	154
	0	327	212	97	88
<u>Experiment E</u>					
	300	206624	151331	229725	203715
	30	117560	96616	138972	81107
	10	14203	7547	12502	8011
	3	815	158	237	203
	0	541	156	140	215



#### 4. DISCUSSION AND SIGNIFICANCE

The potential of the Micronucleus Test to measure genotoxicity has not yet been realized because of variable methodology and inadequate standardization. Also, current applications to epidemiologic studies of disease states have been based upon methodologies of uncertain reproducibility. Current applications, also, have not characterized differences between the mouse erythrocyte model and the human peripheral blood mononuclear leukocyte model. Further differences might be seen in a newer human erythrocyte study being performed in asplenic human subjects (MacGregor). It is unclear the expression of a micronucleus in mouse or human erythrocytes is caused by the same process that is responsible for PBML micronuclei. Immature erythrocytes normally contain a micronucleus that, as the cell matures, is removed by the spleen. Since human PBML micronuclei are seen above background levels only when exposed to a genotoxic initiator and a blastogenic agent, it is entirely possible that the methodologies are not comparable. However, studies that could compare PBML micronuclei and human erythrocyte micronuclei might greatly expand the understanding of the role of the micronucleus in both systems.

Comparison of data without standardization has resulted in conflicting and uncertain results. By contrast, the data presented in this paper, show that the number of micronuclei produced is related to concentration of both the genotoxin (radiation) and the blastogenic agent (PHA). These variables and qualifications have been incorporated into standardized protocols, which generate reproducible results. A major contribution of this study lies in the application of standardized methods to a biologic phenomenon that can be precisely measured. Data between laboratories can be compared and multiple parameters can be studied concurrently.

Information obtained in the course of experiments to standardize the micronucleus assay provided some potentially important insights into the mechanisms of micronucleus formation. Previously, an understanding of mechanisms of micronucleus formation was limited by the methods employed. In some studies specific concentrations of a genotoxic agent were added directly to cells. In other experiments, cells were exposed to radiation. A limited number of studies used blood or bone marrow cells examined from individuals who were exposed to a genotoxin. A common belief based on indirect evidence was that the genotoxin was the major contributor to induction of the micronuclei. Data presented here, which

are based on direct experimental observation, indicate this is not the case. Human peripheral blood mononuclear leukocytes do not produce micronuclei, even after genotoxic exposure, unless they are induced to blast transform.

The current work also documents that micronuclei formation is PHA dose-dependent. No micronuclei are observed in small, nonblast transformed cells. This PHA dose dependency becomes more apparent when PBML have been exposed to a genotoxin, in this study, radiation. Thus, no dose-response relationship exists when PBML are treated with various concentrations of PHA without prior radiation exposure. As an example, Table 4 demonstrates that, without radiation exposure, no greater than 6/1000 cells were ever induced in a culture. However, as many as 80/1000 cells were induced when radiation was combined with PHA.

The importance of the dose-dependent relationship of micronucleus induction with PHA evolves from the recognition that use of too small a concentration of PHA can drastically minimize the ability to identify a substance as genotoxic. Also, the arbitrary use of too great a concentration of PHA can produce the same result. Furthermore, excessively high PHA concentrations produce extensive agglutination, which renders large portions of the slides unreadable.

TABLE 4

## MICRONUCLEI PRODUCTION IN NONIRRADIATED PBML CULTURES CONTAINING PHA

Experiment	PHA (ug/ml)												
	0	3	10	25	30	50	75	100	125	150	175	200	300
A*	0			4.5		4.5	2.0	4.0	1.0	2.5	3.5	5.0	5.0
B	0	0	0		0			1.0					1.5
C	0	0	1.5		1.0			1.0					5.5
D	0	0	0		2.5								3.5
E	0	0.5	0.5		1.5								6.0

\*Donor is a normal, healthy adult without unusual exposures except general anesthesia for a benign fibrotic uterine cyst four months earlier.

An example of the importance of PHA concentration is seen in Figure 14. PBML exposed to 200 R and 30 ug/ml of PHA produced 80/1000 lymphocytes, while the combination of 200 R and 10 ug/ml of PHA produced fewer than 4/1000 lymphocytes. If only 10 ug/ml of PHA had been added to the cultures, it would be reasonable to conclude that 200 R only induced background numbers of micronuclei. In the absence of these precautions, using the micronucleus assay, one could conclude that radiation is not a genotoxin.

The source of the misinformation that led previous investigators to use a single dose of PHA cannot be determined absolutely. Information supplied by PHA manufacturers may have contributed to the use of a single PHA dose. As an example, the package insert from Burroughs-Wellcome recommends that 900 ug/ml of PHA be added to PBML for chromosome analysis (Burroughs-Wellcome).

As shown in this study, the selection of certain concentrations must be based upon experimentation that directly measures what is being studied. Assays based upon the use of a single concentration of PHA, as seen in virtually all published work studying micronuclei, is not sufficient for assessing genotoxicity.

Surprisingly, results of the current studies demonstrate that PHA protects small lymphocytes from the lethal effects of radiation. The absolute dependence of micronuclei formation on PHA stimulation may arise, in part, from its ability to protect cells from the lethal effects of radiation. Lymphoid cells survive because of PHA stimulation, and surviving cells produce micronuclei. Data supporting this hypothesis come from several sources. Cellular debris was uniformly present in gamma-irradiated PBML cultures to which little or no PHA had been added. It was noted consistently that small lymphocytes were selectively destroyed in cultures that contained both blast-transformed and untransformed cells. In these mixed cultures, surviving blast-transformed PBMLs appeared healthy. The relevance of this observation and the possible radioprotective state conferred by blastogenesis are worthy of further study.

It was initially suspected that the drop in numbers of micronuclei at higher concentrations of PHA indicated cell death. Measurement of tritiated thymidine incorporation into DNA was chosen to measure cell function, because of the problems associated with trying to assess viability in massively agglutinated PBML. Figures 9, 12, 15, and 16 display highly reproducible data obtained from 4 separate donors measuring DNA synthesis. In each case maximum DNA synthesis occurred at 30 ug/ml of PHA,

regardless of exposure to radiation. These data indicate that significant radiation exposure (up to 200 R) does not suppress DNA synthesis in human PBML in the first 88 hours following exposure. Also, no decrease in DNA synthesis occurs as PHA concentration increases.

There are several potential explanations for the difference between DNA synthesis and micronuclei production. The absence of radiation-induced depression in DNA synthesis suggests that loss of cell viability is not the explanation for the decreased numbers of micronuclei. It has been suggested (Matter and Jaeger, 1975) that the cell dies following micronucleus formation. The absolute number of PBMLs containing micronuclei is relatively small and would not be noticed in cultures containing high concentrations of PHA that would produce extensive blast transformation. It would be reasonable to expect that cell debris would be present and noticed in cultures of dying cells, once they had produced many micronuclei. Debris was actually noted in cultures exposed to high radiation and that contained low concentrations of PHA.

Micronuclei counting is a static process that may not accurately represent the results of the dynamic events of micronuclei formation and loss. One source of loss, as discussed in the preceding paragraphs, is the death of micronuclei-containing cells. Another possible explanation for the decrease in micronuclei is that, after the

micronucleus is produced, it gradually moves toward the cytoplasmic membrane through which it is released. Micronuclei were observed very close to the main nucleus and well into the cytoplasm. Several in individual cells were observed at the membrane with an apparent bulge in the membrane. Interestingly, no micronuclei were seen outside the cell, nor have extracellular micronuclei been reported. It is uncertain that free micronuclei can be detected microscopically, since it is likely they would appear as debris. It is also questionable as to whether they could exist intact as extracellular entities or whether they would be degraded. Studies have not been performed to characterize the composition of micronuclei; therefore, their susceptibility to degradation is unknown.

The expulsion of the micronucleus from the cell is consistent with a hypothesis that the micronucleus is formed in response to exposure to a genotoxic agent in an attempt to repair the nucleus. Successful repair is dependent upon intensity of damage to the DNA. It has been proposed that the micronucleus represents nuclear material that lagged behind during spindle formation. The nuclear material condenses into a micronucleus following cell division. Direct evidence to support this mechanism is not available in the literature. However, it seems plausible that, with recent evidence of repair



mechanisms in cells, the micronucleus is, in fact, nuclear material that is no longer needed by the cell (Maugh, 1984). Exposure to high concentrations of a genotoxic agent might inevitably lead to cell death, but would be preceded by an attempt at repair.

The observation, on two separate occasions, of micronuclei in mitosis suggest that micronuclei retain some physiologic activity and can function independently from the main nucleus. Two micronuclei in separate experiments were observed in metaphase while the main nuclei of the cells were resting. The cells were intact, and the micronuclei were clearly defined and separated from the nucleus. It is not known if the dividing micronucleus within a resting lymphocyte yields two micronuclei within the cells. Likewise, the fate of "daughter" micronuclei is conjectural at this time. Daughter micronuclei may be expelled as part of a repair process or may represent mortal loss of information to the cell. The appearance of dividing micronuclei is unusual but suggests a much larger genetic role for the micronucleus than the common indicator of cell death. These observations raise questions that include the possibility that the accepted mechanism for production of micronuclei is incorrect and that micronuclei formation may be linked to the hypoploid state of many neoplastic cells.

The current studies also generated some preliminary information regarding gamma interferon production in relation to micronuclei formation by PBML. No gamma interferon was induced by radiation, regardless of the magnitude of exposure, until PHA concentrations of 30 ug/ml or greater were reached. Interferon titers were not affected by radiation, and high titers of interferon were produced by PBML exposed to PHA and 100 R and 200 R. The ability of the cell to produce gamma interferon and synthesize DNA under conditions of radiation-induced micronuclei formation can be interpreted in several ways:

- 4.1.1. Cells that produce micronuclei are not the cells that make interferon and synthesize DNA.
- 4.1.2. The sequence of events resulting in structural changes represented visually as micronuclei are more sensitive indicators of genotoxic damage than either 3HTdr uptake or gamma interferon production.
- 4.1.3. Nuclear damage occurs after the transcription of the interferon genome for DNA has occurred and, therefore, has no effect on gamma interferon synthesis.
- 4.1.4. 3HTdr is incorporated into newly synthesized DNA, as well as radiation-damaged DNA, which falsely suggests no

damage, while interferon production declines as an accurate representation of radiation cell damage.

- 4.1.5. Persistence of an intact interferon response in irradiated PBML cultures suggests that micronuclei formation may occur as part of a physiologic process of repair, as opposed to a pathologic process of cell degeneration and death.

The modified micronucleus assay described in this paper has immediate application in several areas of both basic science and environmental medicine. The challenge to standardize this assay was not in the technical difficulty of the task but in understanding the role of contributing factors and quantitating their effects. With these considerations in mind, the assay has wide application.

The major observations are that micronuclei production is a function of PHA and, by extension, other mitogen concentration; that without exposure to a genotoxic agent followed by stimulation with PHA, micronuclei production is not enhanced; that radiation damage to PBML appears to be markedly diminished with the introduction of PHA to the cultures; and that DNA synthesis is not affected by radiation exposure, regardless of PHA concentration. The task at hand is to use

this information to develop a clearer understanding of what biologic and environmental events will alter micronuclei formation, the physiological significance of micronuclei, the events leading to their formation and their ultimate fate, and whether these events represent repair and eventual return to normal function or to permanent damage and death.

The descriptive methodology for a micronucleus assay, first introduced by Schmid, addressed the real need for accurately quantitating the effect of genotoxins on animals under actual conditions of environmental exposure. The ultimate objective was to be able to assess risks of genotoxic injury to humans. The results of the current studies suggest that modifications of the micronucleus test system can be applied with confidence to the study of genotoxic exposure in humans. Recognition of the importance of mitogen concentration can be used to develop assays that will provide data which can be reproduced between laboratories. These findings have been incorporated into an easily performed assay that uses existing microculture techniques. Acceptance of this approach to micronuclei quantitation will come if the standardization described in this paper can be demonstrated to be reproducible in situations in which its predecessors have failed.

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